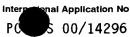## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 150.192W01	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. PCT/US 00/ 14296	International filing date *(day/month/year)* 24/05/2000	(Earliest) Priority Date *(day/month/year)* 24/05/1999	

Applicant MAYO MEDICAL VENTURES

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of _____5_____ sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

    a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

    [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

    b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

    [X] contained in the international application in written form.

    [X] filed together with the international application in computer readable form.

    [ ] furnished subsequently to this Authority in written form.

    [ ] furnished subsequently to this Authority in computer readble form.

    [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

    [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [X] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

    [ ] the text is approved as submitted by the applicant.

    [X] the text has been established by this Authority to read as follows:

    METHODS TO INHIBIT INFECTIOUS AGENT TRANSMISSION DURING XENOTRANSPLANTATION

5. With regard to the **abstract,**

    [X] the text is approved as submitted by the applicant.

    [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

    [ ] as suggested by the applicant.   [X] None of the figures.

    [ ] because the applicant failed to suggest a figure.

    [ ] because this figure better characterizes the invention.

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7   C12N15/48   C12N15/62   C07K14/15   C07K19/00   A61K48/00
         C12Q1/68   C07K16/10   C12N7/01

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7   C12N   C07K   A61K   C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	AKIYOSHI D. E. ET AL.: "Identification of a full-length cDNA for an endogenous retrovirus of miniature swine" JOURNAL OF VIROLOGY, vol. 72, no. 5, May 1998 (1998-05), pages 4503-4507, XP002126425 ISSN: 0022-538X	15-20
Y	the whole document	1-14, 21-28, 30,32-39

-/--

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 September 2000	04/10/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Mandl, B

Form PCT/ISA/210 (second sheet) (July 1992)

5

page 1 of 2

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category°	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	VANBROCKLIN M. ET AL.: "Expression of a murine leukemia virus Gag-Escherichia coli RNase HI fusion polyprotein significantly inhibits virus spread." JOURNAL OF VIROLOGY, vol. 71, no. 4, 1997, pages 3312-3318, XP002148090 ISSN: 0022-538X the whole document	1-14, 21-28, 32-39
X	WO 98 53104 A (MEDICAL RES COUNCIL ;STOYE JONATHAN PAUL (GB); WEISS ROBIN ANTHONY) 26 November 1998 (1998-11-26)	29,31
Y	the whole document	30
A	NATSOULIS G. ET AL.: "NEW ANTIVIRAL STRATEGY USING CAPSID-NUCLEASE FUSION PROTEINS" NATURE, vol. 352, no. 6336, 15 August 1991 (1991-08-15), pages 632-635, XP000608400 ISSN: 0028-0836 the whole document	15-28
A	SUN L.-Q. ET AL.: "Ribozyme-mediated suppression of Moloney murine leukemia virus and human immunodeficiency virus type I replication in permissive cells." PROC. NATL. ACAD. SCI. USA, vol. 91, October 1994 (1994-10), pages 9715-9719, XP002148091 the whole document	13
A	WO 96 32494 A (UNIV ALABAMA RES FOUND) 17 October 1996 (1996-10-17) the whole document	15-28
A	PATIENCE C. ET AL.: "INFECTION OF HUMAN CELLS BY AN ENDOGENOUS RETROVIRUS OF PIGS" NATURE MEDICINE, vol. 3, no. 3, 1 March 1997 (1997-03-01), pages 282-286, XP002037074 ISSN: 1078-8956 the whole document	1-39

5

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

FURTHER INFORMATION CONTINUED FROM   PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-28,32-39

> A method to inhibit or prevent infectious agent transmission in a mammalian transplant recipient; an isolated and purified nucleic acid molecule comprising at least a portion of a pig endogenous retrovirus, the corresponding polypeptides and methods of using them; a method to detect human tropic pig endogenous retroviruses; a fusion protein comprising a capsid or envelope protein of PERV and a degradative enzyme, the corresponding nucleic acid and a host cell comprising it.

2. Claims: 29-31

> An antibody that specifically bings pig endogenous retrovirus.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9853104	A	26-11-1998	AU	7442698 A	11-12-1998
			EP	0981651 A	01-03-2000
			GB	2333102 A	14-07-1999
			US	6100034 A	08-08-2000
WO 9632494	A	17-10-1996	AU	5482896 A	30-10-1996
			CA	2230925 A	17-10-1996
			EP	0873415 A	28-10-1998
			US	6001985 A	14-12-1999

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 150.192WO1	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US00/14296	International filing date *(day/month/year)* 24/05/2000	Priority date *(day/month/year)* 24/05/1999

International Patent Classification (IPC) or national classification and IPC C12N15/48

Applicant MAYO FOUNDATION FOR MEDICAL EDUCATION ... et al.

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

I	☒	Basis of the report
II	☐	Priority
III	☐	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☒	Lack of unity of invention
V	☒	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
VI	☐	Certain documents cited
VII	☐	Certain defects in the international application
VIII	☒	Certain observations on the international application

Date of submission of the demand 13/12/2000	Date of completion of this report 10.07.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Roscoe, R Telephone No. +49 89 2399 2554

Form PCT/IPEA/409 (cover sheet) (January 1994)

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17)):*
    Description, pages:

    1-83   as originally filed

    Claims, No.:

    1-45   as received on   02/01/2001   with letter of   22/12/2000

    Drawings, sheets:

    1/21-21/21   as originally filed

    Sequence listing part of the description, pages:

    1-30, as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

    These elements were available or furnished to this Authority in the following language: , which is:

    ☐   the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
    ☐   the language of publication of the international application (under Rule 48.3(b)).
    ☐   the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

    ☒   contained in the international application in written form.
    ☒   filed together with the international application in computer readable form.
    ☐   furnished subsequently to this Authority in written form.
    ☐   furnished subsequently to this Authority in computer readable form.
    ☐   The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
    ☐   The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

    ☐   the description,   pages:

    ☐   the claims,   Nos.:

    ☐   the drawings,   sheets:

5. ☐   This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

    (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

    ☐   restricted the claims.

    ☐   paid additional fees.

    ☐   paid additional fees under protest.

    ☐   neither restricted nor paid additional fees.

2. ☐   This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

    ☐   complied with.

    ☐   not complied with for the following reasons:

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

    ☒   all parts.

    ☐   the parts relating to claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

    Novelty (N)   Yes: Claims   1-14, 16-28, 32-45

	No:	Claims	15, 29-31
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-45
Industrial applicability (IA)	Yes:	Claims	15, 16, 20-31, 40, 41, 44
	No:	Claims	1-14, 17-19, 32-39, 42, 43, 45

2. Citations and explanations
    see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

The documents mentioned in the present International Preliminary Examination Report are numbered as in the search report, i.e. D1 corresponds to the first document of the search report etc.

IV.   Lack of Unity

The present application lacks unity. The only concept that links detection assays for PERVs and related products (i.e. antibodies) to methods of prevention of PERV (or other infectious agent) transmission from transplant tissues to recipients by introduction of transgenes into the donor cells is PERV. However PERVs were already known in the art (e.g. D1, D3 or D7). Hence, common concept is not even novel. For practical reasons this objection is not persued in the International Phase.

V.   Reasoned statement on Novelty, Inventive Step and Industrial Applicability

- **Novelty (Art.33(2) PCT)**

Claim 15 - anticipated by nucleic acids disclosed in D1, D3 and D7.

Claims 29-31 are anticipated by D3 which suggests making antibody probes for detecting PERV expression (particularly direct probes to N-terminus of env).

- **Inventive Step (Art.33(3) PCT)**

Matter not directly related to inhibition of PERV transfer from xenografts:

Claims 15-17 contain no inventive subject-matter. PERV sequences had already been identified and identification of further sequences by use of probes (see e.g. D1 or D7) is simple (due to extremely high levels of sequence conservation). Reuse of any such further sequences as probes is merely a trivial alternative which does not solve any new problem vis a vis the prior art probes.

Claims 18 and 19 merely relate to transformation of PERV sequences into host cells. It is trivial to insert a known DNA into a cell unless some unexpected

technical effect can be achieved thereby. That is not the case in claims 18 and 19 since proteins not manipulated to be of e.g. medical utility. Hence, claims 18 and 19 are not inventive.

Claims 29-31 contain no inventive subject-matter. Raising antibodies to known proteins is trivial especially in view of possible probe utility (e.g. D3)

<u>Matter relating to inhibition of PERV transfer from xenografts</u>:

(i)   Fusion proteins

The construction of fusion proteins between retroviral proteins and virus inhibitory proteins has been disclosed in the prior art (see D2, D4 or D6). Such fusion proteins could find use for treatment of non-transplant-associated retroviral infection aswell as in applicants proposed xenograft context. Mere application of this technology to an alternative retroviral system of viral system in general cannot be considered inventive. It is at best only the xenograft context which could be considered to impart inventivity and hence, given that product claims per se cannot be limited to this context, mere provision of the fusion proteins of claims 21-26 and systems for expression thereof in claims 27 and 28 cannot be considered inventive.

(ii)   Methods for inhibiting infectious agent transmission

The cited prior art does not suggest the use of transplant donor cells which have been rendered transgenic in an attempt to prevent transmission of infective agents therefrom. D7 is the closest prior art identified in this context. D7 recognizes the problem of PERV transmission to humans during organ transplantation. As a remedy, removal of PERV sequences from the pig genome is suggested (p.285, col.1). Further, (presumably until this has happened) it is stated that precautions should be taken to minimize such zoonosis and to monitor retrovirus infection in early clinical trials. It is not stated how this problem should be addressed. Given this statement, a skilled person is presented with a limited choice of options. These are basically continuous administration of antiretroviral medicaments, or integration of an antiretroviral transgene construct into donor or

recipient tissues (or both). Applicant has chosen to make the donor organ transgenic (the technology used is defined very broadly, and it is noted that no new techniques as such have been introduced). Hence, applicant has made a selection from a very limited number of options to deal with a recognized problem. No surprising effect has been shown, and if it had been this would be limited to a particular method, not any method of donor-targetted transgene based antiretroviral treatment. It is noted that the concept of donor organ genetic manipulation in general is not a novel one. WO-A-94/16065 discloses donor cells with adenoviral E19 protein (reduces MHC I presentation) expressing transgenes, WO-A-95/28412 discloses transgenic swine as organ donors where alpha(1,3)galactosyltransferase expression is prevented by an antisense transgene.

The present application is thus considered devoid of inventive subject-matter.

- **Industrial Applicability (Art.33(4) PCT)**

For the assessment of the present claims 1-14, 17-19, 32-39, 42, 43 and 45 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Claims 1-14, 17-19, 32-39, 42, 43 and 45 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

VIII. **Certain observations**

- **Clarity (Art.6 PCT)**

The number of independent method claims relating to methods of inhibiting infectious agent transmission is excessive and unacceptable (i.e. claims 1, 2, 3, 36, 37, 40, 42).

Claims 1-4 - size of portion not defined so can be any protein. Any protein would not solve problem

Claim 15 - size of portion / hybridizing conditions not defined

Claim 16 "comprising a nucleic acid segment" is unnecessary and renders wording confusing. Further, given that nucleic acid defined in open-ended manner can contain reading frame of any known protein, thus claim effectively lacks novelty over all proteins. Further actual proteins encoded by nucleic acids of Seq.ID nos 18-23 and 32 not identified anywhere in application or claims.

Claim 18 - method makes no sense - seem simply to transform a cell with

Claims 20, 37, 41, 42, 44, 45 - size of portion

Note: It has been noted that claim 41 encompasses transgenic human beings. Such matter would not be admissible in a European Regional Phase.

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

Applicant's or agent's file reference
(if desired) (12 characters maximum)   150.192WO1

Box No. I	TITLE OF INVENTION

METHODS TO INHIBIT INFECTIOUS AGENT TRANSMISSION

Box No. II	APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

MAYO FOUNDATION FOR MEDICAL EDUCATION AND RESEARCH
200 First Street SW
Rochester, Minnesota 55905
United States of America

☐ This person is also inventor.

Telephone No.

Facsimile No.

Teleprinter No.

State *(that is, country)* of nationality:
US

State *(that is, country)* of residence:
US

This person is applicant for the purposes of:
☐ all designated States
☒ all designated States except the United States of America
☐ the United States of America only
☐ the States indicated in the Supplemental Box

Box No. III	FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

FEDERSPIEL, Mark J.
5722 Colonial Lane SE
Rochester, Minnesota 55904
United States of America

This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:
US

State *(that is, country)* of residence:
US

This person is applicant for the purposes of:
☐ all designated States
☐ all designated States except the United States of America
☒ the United States of America only
☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV	AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent   ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*
VIKSNINS, Ann S.
Schwegman, Lundberg, Woessner & Kluth
P.O. Box 2938
Minneapolis, Minnesota 55402
United States of America

Telephone No.
(612) 373-6900

Facsimile No.
(612) 339-3061

Teleprinter No.

☐ **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Form PCT/RO/101 (first sheet) (July 1998; reprint January 2000)   *See Notes to the request form*

PCT

REQUEST

The undersigned requests that the present
international application be processed
according to the Patent Cooperation Treaty.

Applicant's or agent's file reference
(if desired) (12 characters maximum)   150.192WO1

Box No. I	**TITLE OF INVENTION**

METHODS TO INHIBIT INFECTIOUS AGENT TRANSMISSION

Box No. II	**APPLICANT**

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

MAYO MEDICAL VENTURES
200 First Street SW
Rochester, Minnesota 55905
United States of America

[ ] This person is also inventor.

Telephone No.

Facsimile No.

Teleprinter No.

State *(that is, country)* of nationality:
US

State *(that is, country)* of residence:
US

This person is applicant
for the purposes of: [ ] all designated States   [X] all designated States except the United States of America   [ ] the United States of America only   [ ] the States indicated in the Supplemental Box

Box No. III	**FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)**

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

FEDERSPIEL, Mark J.
5722 Colonial Lane SE
Rochester, Minnesota 55904
United States of America

This person is:

[ ] applicant only

[X] applicant and inventor

[ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:
US

State *(that is, country)* of residence:
US

This person is applicant
for the purposes of: [ ] all designated States   [ ] all designated States except the United States of America   [X] the United States of America only   [ ] the States indicated in the Supplemental Box

[ ] Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV	**AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE**

The person identified below is hereby/has been appointed to act on behalf
of the applicant(s) before the competent International Authorities as: [X] agent   [ ] common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*
VIKSNINS, Ann S.
Schwegman, Lundberg, Woessner & Kluth
P.O. Box 2938
Minneapolis, Minnesota 55402
United States of America

Telephone No.
(612) 373-6900

Facsimile No.
(612) 339-3061

Teleprinter No.

[ ] **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Box No.V   DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked)*:

Regional Patent

[X] **AP   ARIPO Patent: GH** Ghana, **GM** Gambia, **KE** Kenya, **LS** Lesotho, **MW** Malawi, **SD** Sudan, **SL** Sierra Leone, **SZ** Swaziland, **TZ** United Republic of Tanzania, **UG** Uganda, **ZW** Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT

[X] **EA   Eurasian Patent: AM** Armenia, **AZ** Azerbaijan, **BY** Belarus, **KG** Kyrgyzstan, **KZ** Kazakhstan, **MD** Republic of Moldova, **RU** Russian Federation, **TJ** Tajikistan, **TM** Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT

[X] **EP   European Patent: AT** Austria, **BE** Belgium, **CH and LI** Switzerland and Liechtenstein, **CY** Cyprus, **DE** Germany, **DK** Denmark, **ES** Spain, **FI** Finland, **FR** France, **GB** United Kingdom, **GR** Greece, **IE** Ireland, **IT** Italy, **LU** Luxembourg, **MC** Monaco, **NL** Netherlands, **PT** Portugal, **SE** Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT

[X] **OA   OAPI Patent: BF** Burkina Faso, **BJ** Benin, **CF** Central African Republic, **CG** Congo, **CI** Côte d'Ivoire, **CM** Cameroon, **GA** Gabon, **GN** Guinea, **GW** Guinea-Bissau, **ML** Mali, **MR** Mauritania, **NE** Niger, **SN** Senegal, **TD** Chad, **TG** Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* ....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

[X] **AE** United Arab Emirates	[X] **LR** Liberia
[X] **AL** Albania	[X] **LS** Lesotho
[X] **AM** Armenia	[X] **LT** Lithuania
[X] **AT** Austria	[X] **LU** Luxembourg
[X] **AU** Australia	[X] **LV** Latvia
[X] **AZ** Azerbaijan	[X] **MA** Morocco
[X] **BA** Bosnia and Herzegovina	[X] **MD** Republic of Moldova
[X] **BB** Barbados	[X] **MG** Madagascar
[X] **BG** Bulgaria	[X] **MK** The former Yugoslav Republic of Macedonia
[X] **BR** Brazil	
[X] **BY** Belarus	[X] **MN** Mongolia
[X] **CA** Canada	[X] **MW** Malawi
[X] **CH and LI** Switzerland and Liechtenstein	[X] **MX** Mexico
[X] **CN** China	[X] **NO** Norway
[X] **CR** Costa Rica	[X] **NZ** New Zealand
[X] **CU** Cuba	[X] **PL** Poland
[X] **CZ** Czech Republic	[X] **PT** Portugal
[X] **DE** Germany	[X] **RO** Romania
[X] **DK** Denmark	[X] **RU** Russian Federation
[X] **DM** Dominica	[X] **SD** Sudan
[X] **EE** Estonia	[X] **SE** Sweden
[X] **ES** Spain	[X] **SG** Singapore
[X] **FI** Finland	[X] **SI** Slovenia
[X] **GB** United Kingdom	[X] **SK** Slovakia
[X] **GD** Grenada	[X] **SL** Sierra Leone
[X] **GE** Georgia	[X] **TJ** Tajikistan
[X] **GH** Ghana	[X] **TM** Turkmenistan
[X] **GM** Gambia	[X] **TR** Turkey
[X] **HR** Croatia	[X] **TT** Trinidad and Tobago
[X] **HU** Hungary	[X] **TZ** United Republic of Tanzania
[X] **ID** Indonesia	[X] **UA** Ukraine
[X] **IL** Israel	[X] **UG** Uganda
[X] **IN** India	[X] **US** United States of America
[X] **IS** Iceland	
[X] **JP** Japan	[X] **UZ** Uzbekistan
[X] **KE** Kenya	[X] **VN** Viet Nam
[X] **KG** Kyrgyzstan	[X] **YU** Yugoslavia
[X] **KP** Democratic People's Republic of Korea	[X] **ZA** South Africa
	[X] **ZW** Zimbabwe
[X] **KR** Republic of Korea	
[X] **KZ** Kazakhstan	Check-boxes reserved for designating States which have become party to the PCT after issuance of this sheet:
[X] **LC** Saint Lucia	[X] **AG** Antigua and Barbuda
[X] **LK** Sri Lanka	[X] **DZ** Algeria

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation (including fees) must reach the receiving Office within the 15-month time limit.)*

Box No. VI   PRIORITY CLAIM ☐ Further priority claims are indicated in the Supplemental Box.

Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is:		
		national application: country	regional application:* regional Office	international application: receiving Office
item (1) (24.05.99) 24 May 1999	60/135,631	US		
item (2)				
item (3)				

☒ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office)* identified above as item(s):   (1)

* Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.

Box No. VII   INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used)* :	Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority):*		
ISA / EP	Date *(day/month/year)*	Number	Country *(or regional Office)*

Box No. VIII   CHECK LIST; LANGUAGE OF FILING

This international application contains the following **number of sheets**:	This international application is **accompanied by the item(s) marked below:**
request : 3	1. ☒ fee calculation sheet
description (excluding sequence listing part) : 83	2. ☐ separate signed power of attorney
claims : 5	3. ☐ copy of general power of attorney; reference number, if any:
abstract : 1	4. ☐ statement explaining lack of signature
drawings : 21	5. ☐ priority document(s) identified in Box No. VI as item(s):
sequence listing part of description : 30	6. ☐ translation of international application into (language):
	7. ☐ separate indications concerning deposited microorganism or other biological material
Total number of sheets : 143	8. ☒ nucleotide and/or amino acid sequence listing in computer readable form
	9. ☒ other *(specify)*: general transmittal; check; return postcard; Comm. re: sequence lisitng

Figure of the drawings which should accompany the abstract: 6	**Language of filing of the international application:** English

Box No. IX   SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

Ann S. Viksnins

── For receiving Office use only ──

1. Date of actual receipt of the purported international application:	2. Drawings:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:	☐ received:
4. Date of timely receipt of the required corrections under PCT Article 11(2):	☐ not received:
5. International Searching Authority (if two or more are competent): ISA /	6. ☐ Transmittal of search copy delayed until search fee is paid.

── For International Bureau use only ──

Date of receipt of the record copy by the International Bureau:

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 150.192WO1	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No. PCT/US 00/ 14296	International filing date *(day/month/year)* 24/05/2000	(Earliest) Priority Date *(day/month/year)* 24/05/1999

Applicant

MAYO MEDICAL VENTURES

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of _____5_____ sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

a. With regard to the **language**, the international search was carried out on the basis of the international application In the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the International application, the international search was carried out on the basis of the sequence listing :

[X] contained in the International application in written form.

[X] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readble form.

[ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [X] **Unity of Invention Is lacking** (see Box II).

4. With regard to the **title,**

[ ] the text is approved as submitted by the applicant.

[X] the text has been established by this Authority to read as follows:

METHODS TO INHIBIT INFECTIOUS AGENT TRANSMISSION DURING XENOTRANSPLANTATION

5. With regard to the **abstract,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract Is Figure No.

[ ] as suggested by the applicant.   [X] None of the figures.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the Invention.

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER				
IPC 7 C12N15/48	C12N15/62	C07K14/15	C07K19/00	A61K48/00
C12Q1/68	C07K16/10	C12N7/01		

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7   C12N C07K A61K C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	AKIYOSHI D. E. ET AL.: "Identification of a full-length cDNA for an endogenous retrovirus of miniature swine" JOURNAL OF VIROLOGY, vol. 72, no. 5, May 1998 (1998-05), pages 4503-4507, XP002126425 ISSN: 0022-538X	15-20
Y	the whole document	1-14, 21-28, 30,32-39

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

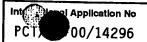| Date of the actual completion of the international search	Date of mailing of the international search report
21 September 2000	04/10/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Mandl, B

Form PCT/ISA/210 (second sheet) (July 1992)

page 1 of 2

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	VANBROCKLIN M. ET AL.: "Expression of a murine leukemia virus Gag-Escherichia coli RNase HI fusion polyprotein significantly inhibits virus spread." JOURNAL OF VIROLOGY, vol. 71, no. 4, 1997, pages 3312-3318, XP002148090 ISSN: 0022-538X the whole document	1-14, 21-28, 32-39
X	WO 98 53104 A (MEDICAL RES COUNCIL ;STOYE JONATHAN PAUL (GB); WEISS ROBIN ANTHONY) 26 November 1998 (1998-11-26)	29,31
Y	the whole document	30
A	NATSOULIS G. ET AL.: "NEW ANTIVIRAL STRATEGY USING CAPSID-NUCLEASE FUSION PROTEINS" NATURE, vol. 352, no. 6336, 15 August 1991 (1991-08-15), pages 632-635, XP000608400 ISSN: 0028-0836 the whole document	15-28
A	SUN L.-Q. ET AL.: "Ribozyme-mediated suppression of Moloney murine leukemia virus and human immunodeficiency virus type I replication in permissive cells." PROC. NATL. ACAD. SCI. USA, vol. 91, October 1994 (1994-10), pages 9715-9719, XP002148091 the whole document	13
A	WO 96 32494 A (UNIV ALABAMA RES FOUND) 17 October 1996 (1996-10-17) the whole document	15-28
A	PATIENCE C. ET AL.: "INFECTION OF HUMAN CELLS BY AN ENDOGENOUS RETROVIRUS OF PIGS" NATURE MEDICINE, vol. 3, no. 3, 1 March 1997 (1997-03-01), pages 282-286, XP002037074 ISSN: 1078-8956 the whole document	1-39

5

FURTHER INFORMATION CONTINUED FROM   PCT/ISA/ 210

This International Searching Authority found multiple (groups of)
inventions in this international application, as follows:

1. Claims: 1-28,32-39

> A method to inhibit or prevent infectious agent transmission
> in a mammalian transplant recipient; an isolated and
> purified nucleic acid molecule comprising at least a portion
> of a pig endogenous retrovirus, the corresponding
> polypeptides and methods of using them; a method to detect
> human tropic pig endogenous retroviruses; a fusion protein
> comprising a capsid or envelope protein of PERV and a
> degradative enzyme, the corresponding nucleic acid and a
> host cell comprising it.

2. Claims: 29-31

> An antibody that specifically bings pig endogenous
> retrovirus.

INTERNATIONAL SEARCH REPORT

Box I   Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

   Although claims 1-14, 19 and 32-39 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
   because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

   see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [X] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   [ ] The additional search fees were accompanied by the applicant's protest.

                             [ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1998)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/ 00/14296

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9853104	A	26-11-1998	AU	7442698 A	11-12-1998
			EP	0981651 A	01-03-2000
			GB	2333102 A	14-07-1999
			US	6100034 A	08-08-2000
WO 9632494	A	17-10-1996	AU	5482896 A	30-10-1996
			CA	2230925 A	17-10-1996
			EP	0873415 A	28-10-1998
			US	6001985 A	14-12-1999

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

CORRECTED VERSION

09/980826

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
30 November 2000 (30.11.2000)

PCT

(10) International Publication Number
WO 00/71726 A1

(51) International Patent Classification[7]: C12N 15/48, 15/62, C07K 14/15, 19/00, A61K 48/00, C12Q 1/68, C07K 16/10, C12N 7/01

(21) International Application Number: PCT/US00/14296

(22) International Filing Date: 24 May 2000 (24.05.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/135,631 24 May 1999 (24.05.1999) US

(71) Applicant (for all designated States except US): MAYO FOUNDATION FOR MEDICAL EDUCATION AND RESEARCH [US/US]; 200 First Street SW, Rochester, MN 55905 (US).

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(74) Agent: ELLINGER, Mark, S.; Fish & Richardson P.C., P.A., Suite 3300, 60 South Sixth Street, Minneapolis, MN 55402 (US).

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— with international search report

(48) Date of publication of this corrected version:
27 June 2002

(15) Information about Correction:
see PCT Gazette No. 26/2002 of 27 June 2002, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHODS TO INHIBIT INFECTIOUS AGENT TRANSMISSION DURING XENOTRANSPLANTATION

(57) Abstract: The invention provides nucleic acid molecules, compositions and methods to inhibit or prevent infectious agent transmission from donor to recipient or recipient to donor during or after transplant.

METHODS TO INHIBIT INFECTIOUS AGENT TRANSMISSION DURING XENOTRANSPLANTATION

5 **Background of the Invention**

The success of solid organ transplantation protocols continues to improve while the availability of donor organs remains limited, leading to a critical shortage (Policy et al., 1996). In 1995, only one-half of the patients on organ transplant waiting lists received transplants. Approximately nine patients per

10 day died while waiting for a suitable organ to become available. Xenotransplantation of animal organs is under consideration as a supplemental approach to alleviate this shortage (Policy et al., 1996). The pig is currently the donor of choice for cells, tissues and vascularized organs used in permanent and transient xenotransplantation treatments (Fishman, 1994). Swine offer a number

15 of advantages as a source for xenotransplantations: economic and animal husbandry issues, the ability to produce transgenic animals, experience as a transplantation model, and the development of specific pathogen-free herds to eliminate known pathogens.

Pig to human organ transplants are discordant. However, several groups

20 have engineered transgenic pig lines in efforts to control hyperacute rejection (HAR) (Lin et al., 1996). Two immunologic factors initiate HAR of xenografts: the interaction of natural xenoreactive antibodies to the Galα1-3Gal carbohydrate (α-Gal) of discordant cell proteins; and the inability of the xenograft complement regulatory proteins to control the recipient's complement

25 system. Several approaches to control HAR are being studied including the ablation of Galα1-3Gal expression (Sandrin et al., 1995), and the design of transgenic pigs which express complement regulatory proteins (e.g., DAF, CD59) which have been shown to be protective against HAR (Diamond et al., 1996; Foder et al., 1994; McCurry et al., 1995; Oldham et al., 1996; Ryan,

30 1995).

Several promising xenotransplantation approaches use pig cells and tissues in protocols where xenotic cells and tissues are not rejected as readily as whole organs: fetal pig neuronal cells producing dopamine have been tested in patients to replace cells destroyed by Parkinson's disease (Deacon et al., 1997);

and fetal pig pancreatic islet cell clusters have been tested in protocols to treat diabetes (Groth et al., 1994). Another xenotransplantation approach has used pig liver and kidney xenografts as temporary support treatments for patients with fulminant organ failure (Breimer et al., 1996; Groth et al., 1994; Makowka et al.,

5   1995). These studies have sparked research in the use of bioartificial organs and whole organ perfusion using xenogeneic tissue for bridging patients prior to transplantation. The early successes of these innovative clinical approaches indicate that xenotransplantation protocols using pig cells, tissues and organs will continue regardless of the results of solid organ xenotransplantation.

10   Besides the expected immunological hurdles that must be overcome, xenotransplantation produces a risk of transmitting infectious disease (xenoses) from the source animal tissue to the recipient, and possibly to the recipient's contacts. Xenotransplantation increases the risk of transferring infectious agents from pigs to humans due to the breach of the normal physical barriers against

15   infectious agents upon introduction of pig cells, tissues or organs; the therapeutic immunosuppression of the recipient to prevent graft rejection which interferes with the mechanisms which defend human cells from infection by zoonotic viruses; and the genetic modifications of pigs, e.g., those being tested to control HAR or modify complement activation, may allow zoonotic viruses to evade

20   immune surveillance or prevent the inactivation of invading enveloped viruses (Bergelson et al., 1995; Dorig et al., 1993; Fishman, 1994; Ward et al., 1994; Weiss, 1998).

Since most vertebrates carry endogenous retroviruses in their germline DNA (Boeke et al., 1997), endogenous retroviruses in xenografts may be an

25   infectious risk to xenotransplant patients. The risks of transmission to humans with endogenous viruses of non-humans are unknown and difficult to assess. Endogenous retroviruses from a variety of animals, including baboons, cats, and mice, can infect human cells (Boeke et al., 1997). The ability of endogenous murine leukemia viruses (MLVs) to be activated and infect human tumor

30   xenografts in immunodeficient mice is well documented (Achong et al., 1976; Beattie et al., 1982; Crawford et al., 1979; Gautsch et al., 1980; Suzuki et al., 1977; Tralka et al., 1983; Wunderi et al., 1979). The observation that the gibbon ape leukemia virus SEATO, a virus most likely derived from an endogenous

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retrovirus of Asian wild mice, can cause chronic myelogenous leukemia in normal juvenile gibbon apes highlights the potential risks of exposure to xenotic endogenous retroviruses (Kawakami et al., 1980; Lieber et al., 1975).

5   There have been several reports of MLV retroviral vector stocks that contain the result from recombination between the vector and endogenous retroviruses in the genome of the helper cell line (Chong et al., 1998; Donahue et al., 1992; Patience et al., 1996; Purcell et al., 1996; Vanin et al., 1994). In one study, 3 of 10 rhesus monkeys developed T-cell lymphomas after autologous transplantation of enriched bone marrow stem cells transduced with a MLV

10   retroviral vector stock containing an array of MLV-related recombinant viruses (Vanin et al., 1994). Finally, the activation of endogenous retrovirus by human tumor xenografts as well as studies of graft versus host reaction *in vivo* and mixed lymphocyte reaction *in vitro* (Hirsch et al., 1970; Hirsch et al., 1972; Levy et al., 1977; Sherr et al., 1974), demonstrate that normally inactive endogenous

15   retroviruses can be induced by xenotransplantation conditions.

Pig endogenous retroviruses (PERVs) released by established pig cell lines were observed in the 1970s, but the host range of these viruses was thought to be restricted to pig cells (Armstrong et al., 1971; Lieber et al., 1975; Moennig et al., 1974; Strandstrom et al., 1974; Todaro et al., 1974). Recently, replication-

20   competent pig endogenous retroviruses (PERV) were identified, and shown to be capable of productively infecting human cells *in vitro* (Wilson et al., 1998; Martin et al., 1998; Patience et al., 1997; Takeuchi et al., 1997; Weiss, 1997). PERVs have been associated with pig lymphomas (Bostock et al., 1973; Frazier, 1985; Moennig et al., 1974; Strandstrom et al., 1974; Suzuka et al., 1985).

25   Although Heneine et al. (1998) and Patience et al. (1998) screened blood lymphocytes and sera from 10 diabetic patients that received porcine fetal islet cells, and 2 renal dialysis patients whose circulation had been linked with pig kidneys extracorporeally, respectively, neither group detected PERVs. However, the risk of viral infection is increased in transplantation by the presence of

30   factors commonly associated with viral activation, e.g., immune suppression, graft-versus-host disease, graft rejection, viral coinfection and cytotoxic therapies. Thus, in immunosuppressed xenotransplant patients, there may be a greater risk that infection would lead to disease.

3

Therefore, there is a need for a method to inhibit or prevent transmission of infectious agents, from donor to recipient and/or recipient to donor, after cell, tissue or organ transplant.

Summary of the Invention

5   The invention provides isolated and purified nucleic acid molecules and methods useful to inhibit or prevent the transmission of an infectious agent during xenogeneic or allogeneic cell, tissue or organ transplant.   Infectious agents include, but are not limited to, viruses, bacteria and parasites. The invention includes prophylactic and therapeutic uses of the nucleic acid molecule

10   of the invention which encodes at least a portion of a polypeptide of the infectious agent or which encodes at least a portion of the receptor employed by the infectious agent to enter the cells of the transplant recipient. It is preferred that the expression of the nucleic acid molecule in a donor cell, tissue or organ inhibits or prevents the transmission of the infectious agent from the donor cells,

15   tissue or organ to a transplant recipient, or, alternatively, the transmission of the infectious agent from the recipient to the donor cell, tissue or organ. Preferred transplant recipients are mammals such as primates, e.g., humans, apes and monkeys, as well as canines, felines, bovines, ovines, swine, and equines.

Preferably, the transmission of the following infectious agents is

20   inhibited or prevented: retroviruses, lentiviruses, herpesvirus, e.g., cytomegalovirus (CMV) and Epstein Barr virus (EBV), and hepatitis viruses, e.g., hepatitis A, B or C. Thus, transplanted organs, e.g., bioengineered organs, tissues, or cells, e.g., stem cells, including allografts, e.g., human-to-human transplants, or xenografts, are genetically modified to inhibit the infection of

25   several major infectious pathogens that limit the success of transplantation (e.g., CMV, EBV, HIV and hepatitis viruses) or the infection by potential pathogens, e.g., endogenous viruses such as PERVs. Preferred nucleic acid molecules useful in the practice of the invention include a nucleic acid molecule which encodes gag, pol, env, protease, and accessory proteins (e.g., Vpr, Vif, Nef, Tat,

30   or Rev) of retroviruses, lentiviruses and spumaviruses; a nucleic acid molecule which comprises a capsid or envelope gene of a herpesviruses, e.g., HSV 1 and 2, EBV and CMV; a nucleic acid molecule which comprises the C gene (nucleocapsid), ORFS/pre-S gene (viral surface glycoproteins), ORFX gene

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(regulatory protein), or ORFP gene (viral polymerase) of Hepadnaviruses, e.g.,
hepatitis B virus; a nucleic acid molecule which comprises the C gene (viral
capsid) or the E1 and/or E2 gene of Flaviviruses, e.g., hepatitis C virus; a nucleic
acid molecule which comprises ORF1, e.g., which encodes Pro (protease), the X
5   gene, the H gene (helicase), the Pol gene (polymerase), or the ORF2 gene, which
codes for capsid, of hepatitis E virus; and a nucleic acid molecule which encodes
the P1 gene, the 1A gene, the 1B gene, the 1C gene, or the 1D gene (capsid and
glycoproteins) of hepatitis A virus. The alteration of human cells and tissue is
preferred for modifying pluripotential cells and in *ex vivo* gene therapy
10   protocols.

        Preferably, viral entry and/or assembly is inhibited. To inhibit viral
entry, receptor interference may be employed. Receptor interference refers to
the expression of a receptor protein for the infectious agent or the ligand thereof,
e.g., a viral ligand such as a viral glycoprotein. Preferably, the genome of the
15   donor cell, tissue or organ is augmented with DNA encoding the viral ligand
thereby preventing infection. Alternatively, or in addition, capsid-targeted viral
inactivation may be employed to inhibit or prevent viral replication. Capsid-
targeted viral inactivation (CTVI), reduces or eliminates the production of
infectious virus by incorporating a degradative enzyme into newly synthesized
20   viral particles, e.g., by expressing a fusion polypeptide comprising a viral
polypeptide and a degradative enzyme. Degradative enzymes such as nucleases,
e.g., RNase H, staphylococcal nuclease or ribozymes (see Marshall et al., 1994,
and U.S. Patent No. 5,811,275), lipases or proteases, may be employed in the
practice of the invention. Preferred fusion polypeptides include fusions of viral
25   capsid, envelope or accessory proteins such as Vif, Vpx, Vpr and Nef, with
degradative enzymes.

        A preferred xenograft for use in the methods of the invention is swine
neuronal cells, pancreatic islet cells, hepatocytes, heart, liver or kidney.
Therefore, the invention provides a method in which the genomic DNA of swine
30   cells, tissue or organ to be transplanted is augmented with a recombinant DNA
molecule encoding a polypeptide of an infectious agent which binds to a cell
surface receptor of a transplant recipient, e.g., a viral glycoprotein, or a fusion
polypeptide comprising at least a portion of an infectious agent polypeptide and

5

a degradative enzyme. Infectious agents in this embodiment of the invention include, but are not limited to, those described in Table 1 of Fishman (1994), which is specifically incorporated by reference herein. In particular, PERVs are useful in CTVI methods to inhibit or prevent human-tropic PERV infection in

5   xenotransplant patients.

As described hereinbelow, human tropic PERVs are produced by activated peripheral blood mononuclear cells. The relative PERV proviral copy number doubled upon passaging of the 1°-293 PERV-infected cells to fresh 293 cells, indicating that passaging the virus may amplify the replication-competent

10   PERV. Only PERV-A *env* sequences were detected in DNA isolated from all three 293/PERV infected cultures by PCR analysis with PERV-A, PERV-B and PERV-C specific *env* primers. The 1°-293/PERV DNA contained a low level of PERV-C *env*. PERV-B *env* was not detected in any culture. Characterization of these PERVs indicated that human-tropic PERVs may be more diverse than

15   previously thought. For example, significant sequence differences were found in the cloned LTR and *env* sequences compared to known PERV sequences. The 293/PERV LTRs contained 71 additional nucleotides compared to the PERV-MSL LTR, and the envelope amino acid sequence contains a PERV-A surface region (SU) sequence but a PERV-C transmembrane (TM) region. These

20   PERVs are useful to determine the tissue tropism of various isolates and to determine the prevalence of anti-PERV antibodies in humans exposed to sources of PERV, e.g., pig slaughterhouse workers and xenotransplant patients such as those having a bioartificial liver with pig hepatocytes, as well as in the methods of the invention.

25   Thus, a vector comprising a nucleic acid molecule of the invention may also be employed to deliver soluble *env* genes to mammalian donor cells, tissues or organs prior to cell, tissue or organ transplantation so that the transplanted cells, tissues or organs are less susceptible to infection by an infectious agent, the genome of which comprises the *env* gene. For example, soluble PERV *env*

30   genes are introduced to donor swine cells, tissues or organs to inhibit infection of the cells, tissues or organs by PERV, e.g., to inhibit infection by PERVs after the virus is reactivated following transplantation.

As also described hereinbelow, two genes encoding an avian leukosis virus (ALV) receptor protein, sTva proteins, *stva* and *stva-mIgG*, were efficiently delivered and broadly expressed by ALV-based retroviral vectors both in cultured cells and in chickens. Both the sTva and sTva-mIgG proteins

5   significantly inhibited ALV(A) infection *in vitro* and *in vivo*. The antiviral effect was specific for ALV(A), consistent with a receptor interference mechanism.

Brief Description of the Figures

Figure 1. Stimulated pig peripheral blood mononuclear cells (PBMCs) produce replication-competent human-tropic PERV. (A) Porcine PBMC were

10   cultured with either phytohemagglutinin (PHA) + phorbol myristate acetate (PMA) (black bars) or PMA + calcium ionophore (grey bars) and the supernatants assayed for RT activity. (B) NIH and Yucatan minipig PBMC stimulated by PHA + PMA were cocultured with ST-IOWA cells. Cell culture supernatants were assayed for RT activity. Background RT values for

15   uninfected ST-IOWA cells have been subtracted from the values shown. NIH (black squares); Yucatan (open circles). (C) NIH minipig PBMC stimulated with PHA + PMA were cocultured with human 293 cells: 293 cells cocultured with live PBMC (closed squares); with lethally irradiated PBMC (open squares). The cell culture supernatants were assayed for RT activity.

20   Figure 2. Schematic overview of the generation of the human 293 cell cultures infected with PERV produced by stimulated NIH minipig primary PBMC.

Figure 3. Relative titers of PERV produced from the infected 293 cell cultures. The titer of MLV-βgal/PERV pseudotyped virus stocks produced from

25   the 1°, 2° and 3°-293/PERV cultures were quantitated on human 293 cells (open bars) and pig ST-IOWA cells (black bars). The assays scored β-galactosidase producing cells (BFU) and were done in triplicate.

Figure 4. Analysis of the relative number of PERV proviruses per cell in the 293/PERV infected cultures. Genomic DNA isolated from human 293 cells

30   and the 293/PERV infected cultures was digested with EcoRI, and the fragments separated by agarose gel electrophoresis, transferred to nitrocellulose, and probed with ^{32}P-labeled PERV *pol* sequences. Two internal proviral DNA fragments hybridized to the PERV *pol* probe as expected. The film was scanned

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and the bands quantitated by Image Quant (Molecular Dynamics, Sunnyvale, CA). The blot was stripped and reprobed with ^{32}P-labeled human GAPDH sequences to normalize for the amount of DNA loaded per lane. After the band densities were normalized, the PERV *pol* level of the 1°-293/PERV DNA was

5   set as 1 copy.

Figure 5. RT activity observed in cocultures of 293/2° virus producer cells and human hematopoietic cell lines. The values shown are of cpm of ^{3}H-TTP incorporated in a reverse transcriptase assay measured in cell supernatants sampled at the times indicated post-coculture of 293/2° virus producer cells with

10   each of the cell lines.

Figure 6. A comparison of the deduced amino acid sequences of the *env* genes of PERV-1.15 (SEQ ID NO:3 encoded by SEQ ID NO:18), PERV-A (Letissier et al., 1997; SEQ ID NO:4) and PERV-C (Akiyoshi et al., 1998; SEQ ID NO:5). A schematic representation of the regions of the PERV-1.15 gene

15   homologous to PERV-A (hatched) and PERV-C (open) genes (top figure). The envelope surface (SU) and transmembrane (TM) glycoprotein regions are also shown. Identical amino acids are denoted by a (.); gaps are denoted (_).

Figure 7. Schematic representation of the cloned PERV sequences. A proposed model for a PERV provirus with estimated locations of the viral

20   domains is shown at the top. The PERV sequences contained in the six unique lambda clones are shown compared to the model. The lamA8 *pol* gene contains a 86 nucleotide deletion (del), and the *env* gene contains a 101 nucleotide insertion (ins) compared to all of the comparable sequences.

Figure 8. A comparison of the nucleotide sequences of two PERV LTRs.

25   The LTR sequence of the published cDNA clone PERV-MSL (SEQ ID NO:7) is compared to the lamA1 LTR sequence (SEQ ID NO:6). Identical bases are denoted by (.); gaps are denoted (_). The estimated location of the major regions of the LTR, U3, R, and U5 are indicated. The sequence of the putative tRNA binding site is also indicated (18 of 18 nucleotides identical to the tRNA^{Pro}

30   binding site).

Figure 9. Schematic representation of the construction of recombinant PERV molecular clones.

Figure 10. Analysis of PERV proteins by SDS-PAGE and Western blot.

(A) Virus was pelleted by ultracentrifugation through a 20% sucrose pad, separated by 12% SDS-PAGE, and visualized by staining with Coomassie brilliant blue. Lane 1, 5 ml 293 supernatant; lane 2, 5 ml 2°-293/PERV

5   supernatant; lane 3, 15 ml 2°-293/PERV supernatant; lane 4, 5 ml DF-1 supernatant; and lane 5, 5 ml avian leukosis virus infected DF-1 supernatant. (B) Viral proteins were prepared and separated by SDS-PAGE as described above, and transferred to nitrocellulose. Lanes 1, 3, 5 and 7, 5 ml 293 supernatant; and lanes 2, 4, 6 and 8, 5 ml 2°-293/PERV supernatant. Lanes 1 and 2 were exposed

10   to 1:1000 dilution of goat anti-GALV capsid antisera; lanes 3 and 4 to 1:1000 goat preimmune sera; lanes 5 and 6 to 1:1000 goat anti-SSAV capsid antisera; and lanes 7 and 8 to 1:1000 goat preimmune sera. The filters were washed and exposed to peroxidase-labeled rabbit anti-goat antibody. The antibody complexes were detected by chemiluminescence, and exposed to film for

15   30 seconds. The PERV CA protein migrates at about 25 kDa.

Figure 11. Schematic of sTva antiviral gene constructs and the ALV-based retroviral vectors.

Figure 12. General procedure for using replication-competent ALV-based retroviral vector system *in vitro* and *in vivo*.

20   Figure 13. sTva-mIgG receptor expression levels in DF-1 cells. The sTva-mIgG protein was immunoprecipitated with goat α-mouse-IgG agarose beads from supernatants (500 μl) of DF-1 cultures infected with the RCASBP(C), RCAS(C) or RCOSBP(C) vectors alone (V) or containing the *stva-mIgG* gene (sTva). The immunoprecipitates were denatured, separated by

25   12% SDS-PAGE, and analyzed by Western transfer. The filter was probed with peroxidase-conjugated goat anti-mouse IgG, and the bound protein-antibody complexes were visualized by chemiluminescence on Kodak X-Omat film. sTva-mIgG protein expressed transiently in human embryonic kidney 293 cells was included as a positive control (+).

30   Figure 14. sTva-mIgG expression in sera of chickens infected with RCASBP vectors. The sTva-mIgG protein was immunoprecipitated from chicken serum (500 μl) and analyzed as described (Figure 13 legend). Lane 1, uninfected control; Lane 2, a RCASBP(B) vector alone infected bird; Landes 3-

4, RCASBP(B)stva-mIgG infected birds; Lane 5, a RCASBP(C) vector alone
infected bird; Lane 6, a RCASBP(C)stva-mIgG infected bird; Lane 7,
RCASBP(C)stva-mIgG infected DF-1 cells as a positive control.

5   Figure 15. Analysis of viral and soluble receptor RNA levels in tissues
of chickens infected with RCASBP(B). The figure shows autoradiograms of 6%
polyacrylamide/7.6 M urea gels used to separate the protected RNA probe
fragments produced in an RNase protection assay with RNA from a bird infected
with RCASBP(B)stva or RCASBP(B)stva-mIgG. RNA was prepared from liver
(L), heart (H), spleen (S), bursa (B), thymus (T), kidney (K), and muscle (M)
10   tissues of each bird. RNA from DF-1 cells infected with the appropriate virus
was included as a positive control (+). RNA from bursa of an uninfected bird
was the negative control (-). ALV(B) *env* RNA protects a 467-nt fragment from
the 522-nt ^{32}P-labeled full-length probe [env(B)]; *stva* RNA protects a 388-nt
fragment from the 498-nt probe (stva); and *stva-mIgG* RNA protects a 363-nt
15   fragment from the 423-nt probe (stva-mIgG). Each assay contained a chicken
GAPDH probe as a control for RNA quality and quantity. GAPDH RNA
protects a 200-nt fragment from the 279-nt GAPDH probe.

Figure 16. Analysis of viral and soluble receptor RNA levels in the bursa
of birds infected with RCASBP(B) vectors. The figure shows an autoradiogram
20   of a 6% polyacrylamide/7.6 M urea gel used to separate the protected RNA
probe fragments produced in an RNase protection assay with RNA from the
bursa of birds infected with RCASBP(B) alone (V), RCASBP(B)stva (S),
RCASBP(B)stva-mIgG (I), or uninfected (U). RNA from DF-1 cells infected
with the appropriate virus was included as a positive control (+). ALV(A) *env*
25   RNA protects a 423-nt fragment from the 483-nt ^{32}P-labeled full-length probe
[env(A)]; ALB(B) *env* RNA protects a 467-nt fragment from the 522-nt probe
[env(B)]; *stva* RNA protects a 388-nt fragment from the 498-nt probe (stva); and
stva-mIgG RNA protects a 363-nt fragment from the 423-nt probe (stva-mIgG).
Each assay contained a chicken GapDH probe as a control for RNA quality and
30   quantity. GAPDH RNA protects a 200-nt fragment from the 279-nt GAPDH
probe. Full-length probes not treated with RNase: lane A, env(A); lane B,
env(B); lane S, stva; lane I, stva-mIgG; and Lane G, GAPDH.

~~Figure 17. Analysis of viral and soluble receptor DNA in tissues of~~

RCASBP(B) infected birds and challenged with RAV-1. Genomic DNA was isolated from liver (L), heart (H), spleen (S), bursa (B), thymus (T), kidney (K), and muscle (M) samples of birds infected with RCASBP(B) alone (I),

5   RCASBP(B)stva (II) and RCASBP(B)stva-mIgG (III) and challenged with RAV-1. DNA isolated from DF-1 cells infected with the appropriate virus was used as a positive control (+). DNA isolated from the bursa of an uninfected control bird was used as the negative control (-). DNA sequences were detected by PCR using specific primer pairs: RAV-1 challenge virus DNA [env(A)] was

10   detected using primers specific for ALV(A) *env* yielding a 937 bp fragment; RCASBP(B) vector DNA [env(B)] was detected using primers specific for ALV(B) *env* yielding a 429 bp fragment; *stva* DNA (stva) and *stva-mIgG* DNA (stva-mIgG) were detected with specific primers yielding fragments of 314 bp and 589 bp respectively. The amplified DNA fragments were separated on 0.8%

15   agarose gels and visualized with ethidium bromide. The molecular weight marker is the 1 Kb plus DNA ladder (Gibco).

Figure 18 A) Nucleotide sequence of env gene of PERV 1.15 (SEQ ID NO:18). B) Nucleotide sequence of PERV sequences in lambda A1 clone (SEQ ID NO:19). C) Nucleotide sequence of PERV sequences in lambda A10 clone

20   (SEQ ID NO:20). D) Nucleotide sequence of PERV sequences in lambda A11 clone (SEQ ID NO:32). E) Nucleotide sequence of PERV sequences in lamba A3A clone (SEQ ID NO:21). F) Nucleotide sequence of PERV sequences in lambda A6 clone (SEQ ID NO:22). G) Nucleotide sequence of PERV sequences in lambda clone A8 (SEQ ID NO:23).

25   **Detailed Description of the Invention**

Definitions

As used herein, the terms "isolated and/or purified" refer to *in vitro* preparation, isolation and/or purification of a nucleic acid molecule or polypeptide (e.g., antibody) of the invention, so that it is not associated with *in*

30   *vivo* substances. Thus, with respect to an "isolated nucleic acid molecule", which includes a polynucleotide of genomic, cDNA, or synthetic origin or some combination thereof, the "isolated nucleic acid molecule" (1) is not associated with all or a portion of a polynucleotide in which the "isolated nucleic acid

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molecule" is found in nature, (2) is operably linked to a polynucleotide which it
is not linked to in nature, or (3) does not occur in nature as part of a larger
sequence. An isolated nucleic acid molecule means a polymeric form of
nucleotides of at least 10 bases in length, either ribonucleotides or

5   deoxynucleotides or a modified form of either type of nucleotide. The term
includes single and double stranded forms of DNA. The term "oligonucleotide"
referred to herein includes naturally occurring, and modified nucleotides linked
together by naturally occurring, and non-naturally occurring oligonucleotide
linkages. Oligonucleotides are a polynucleotide subset with 200 bases or fewer

10   in length. Preferably, oligonucleotides are 10 to 60 bases in length and most
preferably 12, 13, 14, 15, 16, 17, 18, 19, or 20 to 40 bases in length.
Oligonucleotides are usually single stranded, e.g., for probes; although
oligonucleotides may be double stranded, e.g., for use in the construction of a
variant. Oligonucleotides of the invention can be either sense or antisense

15   oligonucleotides. The term "naturally occurring nucleotides" referred to herein
includes deoxyribonucleotides and ribonucleotides. The term "modified
nucleotides" referred to herein includes nucleotides with modified or substituted
sugar groups and the like. The term "oligonucleotide linkages" referred to herein
includes oligonucleotides linkages such as phosphorothioate,

20   phosphorodithioate, phophoroselenoate, phosphorodiselenoate,
phosphoroanilothioate, phosphoraniladate, phosphoroamidate, and the like. An
oligonucleotide can include a label for detection, if desired.

The term "isolated polypeptide" means a polypeptide encoded by cDNA
or recombinant RNA, or is synthetic origin, or some combination thereof, which

25   isolated polypeptide (1) is not associated with proteins found in nature, (2) is free
of other proteins from the same source, e.g., free of human proteins, (3) is
expressed by a cell from a different species, or (4) does not occur in nature.

The term "sequence homology" means the proportion of base matches
between two nucleic acid sequences or the proportion amino acid matches

30   between two amino acid sequences. When sequence homology is expressed as a
percentage, e.g., 50%, the percentage denotes the proportion of matches over the
length of a sequence that is compared to some other sequence (the reference
sequence). Gaps (in either of the two sequences) are permitted to maximize

matching; gap lengths of 15 bases or less are usually used, 6 bases or less are
preferred with 2 bases or less more preferred. When using oligonucleotides as
probes or treatments, the sequence homology between the target nucleic acid and
the oligonucleotide sequence is generally not less than 17 target base matches

5   out of 20 possible oligonucleotide base pair matches (85%); preferably not less
than 9 matches out of 10 possible base pair matches (90%), and more preferably
not less than 19 matches out of 20 possible base pair matches (95%).

The term "selectively hybridize" means to detectably and specifically
bind. Polynucleotides, oligonucleotides and fragments of the invention

10   selectively hybridize to nucleic acid strands under hybridization and wash
conditions that minimize appreciable amounts of detectable binding to
nonspecific nucleic acids. High stringency conditions can be used to achieve
selective hybridization conditions as known in the art and discussed herein.
Generally, the nucleic acid sequence homology between the polynucleotides,

15   oligonucleotides, and fragments of the invention and a nucleic acid sequence of
interest is at least 65%, and more typically with preferably increasing
homologies of at least about 70%, about 90%, about 95%, about 98%, and
100%. Preferred PERV nucleic acid sequences for use in the methods of the
invention are those which have at least about 80%, more preferably 90%, and

20   even more preferably 95%, contiguous nucleotide sequence homology or identity
to SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID
NO:22, SEQ ID NO:23 or SEQ ID NO:32. Preferably, PERV nucleic acid
sequences are those which hybridize under moderate, preferably under stringent,
hybridization conditions to SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20,

25   SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23 or SEQ ID NO:32.

Two amino acid sequences are homologous if there is a partial or
complete identity between their sequences. For example, 85% homology means
that 85% of the amino acids are identical when the two sequences are aligned for
maximum matching. Gaps (in either of the two sequences being matched) are

30   allowed in maximizing matching; gap lengths of 5 or less are preferred with 2 or
less being more preferred. Alternatively and preferably, two protein sequences
(or polypeptide sequences derived from them of at least 30 amino acids in
length) are homologous, as this term is used herein, if they have an alignment

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score of at more than 5 (in standard deviation units) using the program ALIGN

with the mutation data matrix and a gap penalty of 6 or greater. See Dayhoff, M.

O., in Atlas of Protein Sequence and Structure, 1972, volume 5, National

Biomedical Research Foundation, pp. 101-110, and Supplement 2 to this

5   volume, pp. 1-10. The two sequences or parts thereof are more preferably

homologous if their amino acids are greater than or equal to 50% identical when

optimally aligned using the ALIGN program.

The term "corresponds to" is used herein to mean that a polynucleotide

sequence is homologous (i.e., is identical, not strictly evolutionarily related) to

10   all or a portion of a reference polynucleotide sequence, or that a polypeptide

sequence is identical to a reference polypeptide sequence. In contradistinction,

the term "complementary to" is used herein to mean that the complementary

sequence is homologous to all or a portion of a reference polynucleotide

sequence. For illustration, the nucleotide sequence "TATAC" corresponds to a

15   reference sequence "TATAC" and is complementary to a reference sequence

"GTATA".

The following terms are used to describe the sequence relationships

between two or more polynucleotides: "reference sequence", "comparison

window", "sequence identity", "percentage of sequence identity", and

20   "substantial identity". A "reference sequence" is a defined sequence used as a

basis for a sequence comparison; a reference sequence may be a subset of a

larger sequence, for example, as a segment of a full-length cDNA or gene

sequence given in a sequence listing, or may comprise a complete cDNA or gene

sequence. Generally, a reference sequence is at least 20 nucleotides in length,

25   frequently at least 25 nucleotides in length, and often at least 50 nucleotides in

length. Since two polynucleotides may each (1) comprise a sequence (i.e., a

portion of the complete polynucleotide sequence) that is similar between the two

polynucleotides, and (2) may further comprise a sequence that is divergent

between the two polynucleotides, sequence comparisons between two (or more)

30   polynucleotides are typically performed by comparing sequences of the two

polynucleotides over a "comparison window" to identify and compare local

regions of sequence similarity.

A "comparison window", as used herein, refers to a conceptual segment of at least 20 contiguous nucleotides and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) of 20 percent or less as compared to the reference sequence

5   (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith and Waterman (1981) Adv. Appl. Math. 2: 482, by the homology alignment algorithm of Needleman and Wunsch (1970) J. Mol. Biol. 48: 443, by the search

10   for similarity method of Pearson and Lipman (1988) Proc. Natl. Acad. Sci. (U.S.A.) 85: 2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by inspection, and the best alignment (i.e., resulting in the highest percentage of

15   homology over the comparison window) generated by the various methods is selected.

            The term "sequence identity" means that two polynucleotide sequences are identical (i.e., on a nucleotide-by-nucleotide basis) over the window of comparison. The term "percentage of sequence identity" means that two

20   polynucleotide sequences are identical (i.e., on a nucleotide-by-nucleotide basis) over the window of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U, or I) occurs in both sequences to yield the number

25   of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. The terms "substantial identity" as used herein denote a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that

30   has at least 85 percent sequence identity, preferably at least 90 to 95 percent sequence identity, more usually at least 99 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 nucleotide positions, frequently over a window of at least 20-50 nucleotides,

wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the window of comparison.

5   As applied to polypeptides, the term "substantial identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least about 80 percent sequence identity, preferably at least about 90 percent sequence identity, more preferably at least about 95 percent sequence identity, and most preferably at least about 99

10   percent sequence identity.

Moderate and stringent hybridization conditions are well known to the art, see, for example sections 9.47-9.51 of Sambrook et al. (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989). For example, stringent conditions are those that (1) employ low ionic

15   strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate (SSC); 0.1% sodium lauryl sulfate (SDS) at 50°C, or (2) employ a denaturing agent such as formamide during hybridization, e.g., 50% formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM

20   NaCl, 75 mM sodium citrate at 42°C. Another example is use of 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 μg/ml), 0.1% sodium dodecylsulfate (SDS), and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC and 0.1%

25   SDS.

CTVI

The present invention describes a method of interfering with viral replication. For example, the fusion of an enzyme (such as a nuclease, lipase, or protease) to a viral capsid, glycoprotein or accessory polypeptide which is

30   incorporated into virions can inactivate essential viral components, such as nucleic acid, protein or lipid, which are necessary for replication. This technique is referred to as capsid-targeted viral inactivation (CTVI), although it includes the targeting of non-capsid fusions, e.g., env fusions, to the virion as well.

The present invention uses nucleases, proteases, and/or lipases as antiviral therapeutic agents that degrade or modify essential viral components, such as nucleic acids, proteins, or lipids. The method of the present invention can be applied to viruses that have relatively flexible capsid structures such as

5   retroviruses, lentiviruses, herpesviruses, poxviruses, togaviruses, hepadnaviruses, caulimoviruses, myxoviruses and paramyxoviruses, that readily allow the encapsidation of foreign proteins in an aqueous internal compartment containing the viral nucleic acid. Preferred viruses to be inhibited by the methods of the invention include porcine endogenous retroviruses (PERVs),

10   herpesviruses such as EBV and CMV, and hepatitis viruses, e.g., hepatitis A, B, and C. See Fields, Virology, Lippincourt-Raven, Philadelphia, PA (1996), which is specifically incorporated by reference herein. The enzyme portion of the fusion protein preferably is oriented so that it is internal to the assembled capsid structure. However, externally facing enzymes may be used in the case of

15   proteases and lipases. The use of such constructs does not require the virus to have an aqueous internal compartment. In the case of lipases, it is desirable to target the enzyme to the envelope of the virus. The enzyme portion can be either inside or outside the viral envelope. In this way, any enveloped virus can be targeted by CTVI.

20   Retroviruses, and the closely related retrotransposons, which are referred to herein generically as retroviruses, are particularly susceptible to CTVI because of their assembly mechanism. It is possible to take advantage of the knowledge of how these capsids assemble to direct a destructive enzyme molecule to the inside surface of the capsid, where it can contract viral nucleic acids or proteins.

25   Retroviral and retrotransposon reverse transcriptases are expressed as natural fusion proteins, in which the polymerase protein forms the C-terminal portion of the primary translation product and the gag protein forms the N-terminal portion. The mechanisms by which Gag-Pol fusion proteins are formed in different retroviruses vary, sometimes involving frameshifting during translation,

30   suppression of a nonsense codon, or splicing. Regardless of the mechanism of synthesis, the reverse transcriptase protein sequence is found at the C-terminus of the Gag protein sequences. In addition, the reverse transcriptase is found internal to the capsid structure. Substitution of a destructive enzyme coding

region for the reverse transcriptase gene (i.e., inserting an open reading frame for a destructive enzyme into the *pol* gene in frame), leads to the assembly of the enzyme in retroviral particles. Further, the nuclease or protease is oriented relative to the capsid in such a way as to have access to the viral RNA or reverse

5   transcriptase. Gag-degradative enzyme fusions, as well as gag-pol-degradative enzyme fusions and env-degradative enzyme fusions, are envisioned. Further, the amino acid linkage between the viral proteins and the degradative enzyme may include sequences that are susceptible to proteases present in the virion, and so are cleaved to yield a soluble degradative enzyme.

10   Thus, a dominant negative effect is obtained by fusion of a protein with a destructive or modifying enzymatic activity that can alter or destroy an essential component of the virus (e.g., DNA, RNA or protein). The destructive or modifying activity is targeted to the desired site of action (inside or closely adjacent to the virus particle) because it is covalently linked to a core protein of

15   the viral particle, e.g., in the same manner as reverse transcriptase.

Besides its versatility, one of the main advantages of CTVI is that it is enzymatic. It does not rely on massive over-expression of a mutant protein that in some way interferes with the viral life cycle, as is the case in the strategies referenced above. Rather, a small number of enzyme molecules (e.g., nucleases)

20   are incorporated into a viral particle, and they cleave or modify one or more essential components of the virus.

CTVI also has advantages over traditional chemotherapy. Viruses, especially viruses with error-prone replicative mechanisms such as HIV and other retroviruses, are likely to become resistant to a chemotherapeutic agent.

25   For example, HIV isolates resistant to AZT, a widely used anti-HIV drug, have been reported. Clearly, resistance to drugs is an important problem with viral infections just as it is for bacterial infections. Gene therapy or intracellular immunization strategies may also be subject to problems of resistance. For example, if a virus mutates such that the capsid protein no longer assembles with

30   the capsid fusion protein of the present invention, the virus will become resistant to the particular CTVI therapy. However, the DNA of the mutant virus can be cloned and the mutant capsid gene can be fused to the gene encoding a nuclease or other enzyme, thereby restoring the efficacy of the therapy. This approach is

not possible with normal drug therapy; once a virus becomes resistant to a given drug, use of that drug has to be abandoned.

Fusion proteins according to the present invention comprise a destructive enzyme which is capable of destroying the replication ability of a virus or virus-

5 like particle. As used in the present invention, "replication ability" encompasses infectivity of a virus particle or transposition of a retrotransposon. Typically, the destructive enzyme is a nuclease, lipase or protease. Protein-modifying enzymes, such as kinases, which diminish enzyme activity can also be used, for example, one which inactivates a viral encoded polymerase. Fusion proteins

10 also comprise a capsid protein, such as Gag, which confers upon the fusion proteins the ability to co-assemble into a virus particle or envelope protein. As mentioned above, it may be desirable in the case of lipase fusions that the virus-encoded portion of the fusion protein be an envelope protein, rather than a capsid protein.

15   Nucleases which can be used in the practice of the present invention to make fusions include restriction endonucleases such as EcoRI, and less specific RNAses, such as T1 RNase, barnase, RNase H1, RNase III, retroviral RNase H domains from reverse transcriptase, and staphylococcal nuclease (SN). Other destructive enzymes which can be used in the practice of the present invention

20 include proteases, e.g., nonhomologous retroviral proteases, and lipases. Any such enzymes known in the art can be used. These enzymes degrade proteins and lipids which may be essential for the replication (infectivity) of particular viruses. Other enzymes which modify protein structures may be used, if they result in the inactivation of proteins which are necessary for the viral life cycle.

25 such protein modification enzymes include: kinases, glycosylases, phosphatases, methyl transferases, acetylases, acylases, farnesyl transferases, and demyristylases.

High levels of expression of the fusion proteins are not required, as a single active enzyme molecule can be sufficient to inactivate a virus particle.

30 However, it is desirable that each virus particle formed have at least a single molecule of the fusion protein. Thus, statistical considerations would dictate that expression at a rate greater than one molecule of fusion protein per virus particle be obtained.

~~In some embodiments of the invention, it may be desirable to utilize~~
conditionally active or marginally active enzymes, since the destructive activity
of the enzyme may be deleterious to cells used to prepare the constructs of the
present invention. Thus, for example, calcium dependent enzymes may be used,

5   which require a threshold level of calcium for activity. Calcium-dependent
destructive enzymes which may be used include calpain, phospholipase A2 and
staphylococcal nuclease. It may also be desirable to non-conditionally diminish
the activity of the destructive enzyme to a level which is not toxic to cells but
which is toxic to viruses. Random mutagenesis can be used to accomplish this

10   end.

DNA molecules are also provided according to the invention, which code
for fusion proteins as described above. The DNA molecules comprise
appropriate transcriptional and translational control signals which allow the
DNA molecules to be expressed in the host cells of the viruses from which the

15   capsid or envelope protein is derived. The expression of the fusion proteins may
be dependent upon the presence of virus in the cell.

Recombinant virus is also provided by the present invention. These
particles comprise a nucleic acid molecule encoding a fusion protein, as
described above. Thus, infection of cells with these recombinant viruses will

20   yield fusion proteins. The recombinant virus is desirably not related to the virus
from which the capsid or envelope protein of the fusion protein is derived. The
recombinant virus is a mere vehicle for introduction of the fusion constructs.
When the cells are infected with viruses which are cognate to the fusion protein
capsid or envelope portion, the normal infection cycle of the viruses proceeds.

25   However, when the viruses encapsidate viral nucleic acid, wild-type capsid or
envelope proteins co-assemble with fusion proteins. Thus, the newly assembled
viruses are hybrids comprising wild-type and fusion capsid or envelope proteins.
The newly assembled viruses are not infective because the enzyme portion of the
fusion protein inactivates either the viral nucleic acid or the essential viral-

30   packaged proteins or lipid comprising the viral envelope.

Exemplary nucleic acid molecules are those comprising PERV nucleic
acid sequences. Thus, for fusion proteins of the invention, it is preferred that
nucleic acid molecules encoding PERV capsid or env are employed. For

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~~example, recombinant virus may be obtained from cells, e.g., helper cells,~~
transfected or infected with a recombinant DNA molecule encoding a fusion
protein comprising PERV capsid linked to a degradative enzyme. Preferably,
once the fusion protein is packaged into virions, the degradative enzyme is
5   proteolytically cleaved from the fusion protein, i.e., it is soluble within the
virion.

        A method of inhibiting virus replication or infectivity is also provided by
the present invention. A nucleic acid molecule encoding a fusion protein is
introduced into a cell susceptible to a virus. The fusion protein may comprise a
10   capsid or envelope protein of the virus as well as a destructive enzyme as
described above. The method of introducing the nucleic acid molecule into the
cell is not critical to the invention, and many such methods are known in the art.
These include, without limitation: infection, transformation, transfection,
lipofection, tungsten microprojectiles, electroporation, cell fusion, and
15   transduction.

        Nucleic acid molecules encoding the fusion protein of the present
invention can be introduced into solid organs, tissues or cells, e.g., stem cells of
the hematopoietic lineage or B lymphocytes. T-lymphocytes and
monocytes/macrophages, which are derived from hematopoietic stem cells, are
20   the primary target of viruses such as HIV. Bone marrow cells can be taken from
an individual, and if desired, the stem cells can be purified from the mixed
population of marrow cells. The nucleic acid molecules of the present invention
can be introduced into the stem cells by means of transfection, or any other
means known in the art. The transfected stem cells can then be reinfused into the
25   individual (autologous stem cell transplantation). To facilitate proliferation of
the transplanted stem cells, the individual's bone marrow can be partially cleared
by irradiation or chemotherapy.

        In another embodiment, the nucleic acid molecule of the present
invention is introduced into an animal, or gametic cells or embryos. Techniques
30   such as microinjection or transfection can be used, as is well known to the art.
The nucleic acid-treated cells can be used in artificial insemination (gametes) or
can be reimplanted into a hormone-prepared female animal (embryos) as is
known in the art.

In yet another method of introducing the nucleic acids of the present invention into cells or an animal, a recombinant virus can be used. The recombinant virus is desirably derived from a virus having the same cell-type tropism as the virus from which the capsid or envelope protein of the gene fusion

5   is derived. Thus, for example, in order to introduce a gene fusion which employs a PERV capsid protein gene, a PERV viral vector may be used. The recombinant virus is then administered according to a route of viral infection for the particular virus of the vector.

An alternative way for introducing the fusion protein encoding construct

10   of the present invention into cells and animals is by means of hybrid virus particles which contain the nucleic acid of the present invention but do not contain the fusion protein of the invention. Hybrid viruses can be prepared which contain the nucleic acid encoding the fusion protein of the present invention using a packaging cell line. Such cell lines may be propagated in

15   culture according to techniques well known in the art. Such cell lines produce the proteins necessary for packaging viral genomes. The nucleic acids of the present invention can be introduced into such a packaging cell line. Desirably, the nucleic acids will contain the packaging signal which is recognized by the packaging proteins of the cell line. Preferably, the fusion protein gene is under

20   the control of an inducible promoter, such as the metallothionine promoter. When it is desirable to produce viral particles carrying the nucleic acid of the present invention, the inducible promoter can be induced to turn on transcription of the fusion protein gene construct, and a protein synthesis inhibitor, such as cycloheximide, can be added so that the fusion protein transcript is not translated

25   in the packaging cell line. Thus, the fusion protein gene construct is transcribed and packaged into the particles produced by the cell line, without interference from (co-assembly with) the fusion protein. When the packaged particles are used to infect cells, the nucleic acid encoding the fusion protein is expressed, and the fusion protein becomes co-assembled into the progeny particles, rendering

30   them non-infectious.

Methods of producing animals, e.g., humans or other non-human animals, which are resistant to a virus are also provided by the present invention. A nucleic acid molecule which encodes a fusion protein as described above is

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introduced into the germ line of an animal or an embryo, or to the animal, e.g.,
via DNA immunization or by infection with recombinant virus. Methods for the
introduction of nucleic acid molecules to animals are known in the art (see, for
example, Salter et al., 1991; Salter et al., 1989; Henininghausen, 1990; Ebert et

5   al., 1991; Wright et al., 1991; Jaenish, 1988; Wagner et al. (U.S. Patent No.
4,873,191); Donahue et al., 1992; Rogers et al., 1993). All somatic cells
resulting from development of zygotes containing the nucleic acid molecule are
resistant to the virus from which the fusion protein capsid or envelope portion is
derived. Methods for making transgenic animals which carry foreign genes in

10   each cell of the animal are known in the art.

Receptor Interference (RI)

Another antiviral strategy targets the first step in the virus life cycle, the
interaction of the viral envelope glycoprotein(s) with a host cell receptor. For
example, retroviral infection of cells can be significantly reduced by expressing

15   soluble forms of the host receptor, which bind the glycoproteins of the incoming
virions before the virion can bind to the host receptor, or the viral envelope
glycoproteins (the product of the *env* gene), which block the host receptors,
directly preventing virion binding.

There are at least two distinguishable steps in viral entry; the first

20   involves recognition and binding of the viral envelope glycoprotein to the host
receptor, the second involves steps that lead to the fusion of the host and viral
membranes. The *env* gene of retroviruses and lentiviruses encodes a polyprotein
precursor that is subsequently processed into two glycoproteins, the surface
glycoprotein (SU) which contains the domains that interact with the host

25   receptor, and the transmembrane glycoprotein (TM) that anchors the SU protein
to the membrane and appears to be directly involved with the fusion between the
virus and cell membranes. Following membrane fusion, the viral core is released
into the cytoplasm where the single-stranded RNA genome found in the virion is
copied into double-stranded DNA (dsDNA) by the viral enzyme reverse

30   transcriptase (RT). This reverse transcription process produces a linear dsDNA
that is longer than the RNA genome from which it derives. The ends of the
linear DNA contain terminally redundant sequences known as long terminal
repeats (LTRs). Retroviruses, and vectors that derive from them, appear to

require the breakdown of the nuclear membrane that occurs during mitosis for
the viral DNA to gain access to the cellular genome. Once this occurs, the linear
viral DNA is inserted into the host genome by the virally encoded enzyme
integrase (IN). The integrated viral DNA is called a provirus. Efficient
5   production of progeny viruses requires the integration of the viral genome into
host DNA.

Cells and animals engineered to express envelope glycoproteins have
been shown to be highly resistant to that strain of retrovirus and to have less
virus-associated pathogenesis (Federspiel et al., 1989; Nihrane et al., 1996;
10   Salter et al., 1989). Based on the receptor interference of endogenous ALV(E),
Salter and Crittenden (1989) proposed that insertion of the ALV(A) envelope
gene into the germline of chickens and its subsequent expression would provide
resistance to infection by ALV(A). Their initial germline studies produced 23
lines of transgenic chickens using ALV-based retroviral vectors and proved two
15   major points. First, these studies showed that avian retroviruses can be
artificially inserted into the germline of chickens at a reasonable efficiency,
stably inherited by subsequent generations, and that the viral genes were
expressed (Crittenden et al., 1989; Salter et al., 1987). Second, these studies
showed that expression of the inserted subgroup (A) envelope glycoproteins
20   could inhibit subsequent ALV(A) infection by subgroup-specific receptor
interference, both *in vitro* and *in vivo* (Salter et al., 1989; Salter et al., 1991).

Two transgenic chicken lines, *alv6* and *alv11*, that did not produce
infectious virus were extensively characterized at the RNA level in different
tissues and at different stags of development. Viral RNA was expressed by both
25   lines in all tissues tested regardless of the stage of development; insert *alv6*
expressed moderate levels of *env* RNA, while insert *alv11* expressed low levels
of *gag* and *env*. Both lines, however, expressed much higher levels of viral RNA
in the tissues associated with ALV pathogenesis, the bursa, thymus, and spleen.
In vitro, both *alv6* (about 4400-fold) and *alv11* (about 11-fold) chicken embryo
30   fibroblasts (CEF) were resistant to ALV(A) infection. The susceptibilities of the
alv6 and *alv11* transgenic lines to ALV(A) infection and associated pathogenesis
were compared to other properties (e.g., resistance measured *in vitro, env*
expression levels, etc.) to determine which characteristics were important

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indicators of an antiviral effect *in vivo*. None of the *alv6* birds were productively

infected as determined by the absence of both infectious ALV(A) and ALV(A)

neutralizing antibodies, while two-thirds of the *alv11* birds were productively

infected by ALV(A). However, neither the *alv6* nor the *alv11* chickens showed

5   significant ALV-induced pathogenesis. Thus, the level of Env glycoprotein

expressed in the target tissues for ALV pathogenesis determined the magnitude

of ALV interference (Federspiel et al., 1991).

Reticuloendotheliosis virus (REV) is very cytotoxic to avian cells *in vitro*

presumably due to the fusionogenic properties of the envelope glycoproteins,

10   especially the TM glycoprotein. REV stocks are therefore produced in D17

cells, a canine osteosarcoma line tolerant to REV infection. D17 cell lines

expressing the REV envelope glycoproteins were significantly more resistant to

REV infection (up to 25,000-fold) compared to controls. However, when the

same REV envelope expression cassette was delivered into chicken embryo

15   fibroblasts (CEF), the REV envelope gene sustained large deletions and the

envelope proteins were not expressed due to cytotoxicity of the REV envelope

glycoproteins to CEF cells. Thus, to reduce potential cytotoxic effects, it is

preferred that soluble forms of viral envelope glycoprotein are employed. The

soluble protein differs from the wild-type protein in that it lacks the

20   transmembrane (TM) domain which anchors the protein in the membrane, and

are therefore secreted from the cell.

The invention will be further described by the following examples.

Example 1

Expression of a Mo-MLV Gag-*E. coli* RNase H Fusion Polyprotein

25   A gene encoding a Mo-MLV Gag-*E. coli* RNase HI fusion was

constructed by linking the RNase HI coding region to the Mo-MLV Gag

polyprotein six codons upstream of the Gag termination codon (Van Brocklin et

al., 1997). The RNase HI coding sequence was isolated from *E. coli* DNA by

PCR amplification with a 5' primer containing a BglII site (5'-GCG CAT GCA

30   GAT CTG ATG CTT AAA CAG GTA GAA ATT TTC ACC GAT GG-3'; SEQ

ID NO:1) and a 3' primer containing a SalI site (5'-GCT GCT GCG TCG ACT

TAA ACT TCA ACT TGG TAG CCT GTA TCT TCC-3'; SEQ ID NO:2). The

reagents and conditions used for the PCR were as described in Federspiel et al.

(1996). The *E. coli* RNase HI fragment was sequenced and contained one silent nucleotide difference from the sequence published by Kanaya and Crouch (1983), at nucleotide 470 (A→G). The Mo-MLV Gag-RNase H fusion construct was based on the design of the Mo-MLV Gag-SN fusion which has been used to

5   introduce SN into virions (Natsoulis et al., 1995).

The genes encoding the CTVI constructs were delivered into CEF by the RCASBP(A) retroviral vector and expressed under the control of the viral promoter-enhancer in the long terminal repeat. CEF were cultured and passaged every 2 days when they reached confluence (Federspiel et al., 1994). ALV

10   retroviral vector propagation was initiated by transfection of plasmid DNA that contained the retroviral vector in a proviral form by the calcium phosphate precipitation method (Kingston et al., 1989). The course of the retroviral infection was monitored by assaying culture supernatants from confluent cells for the viral Gag protein. Three plasmids were used for comparison: Mo-MLV

15   Gag-SN plasmid pGN1600 (Natsoulis et al., 1995); Mo-MLV Gag plasmid pGN1601 (Natsoulis et al., 1995), which produces only the Mo-MLV Gag polyprotein; and Mo-MLV Gag-SN* plasmid pGS293 (Schumann et al., 1996), which differs from Mo-MLV Gag-SN in two missense mutations in the SN gene that result in an inactive SN enzyme. Maximum levels of Mo-MLV Gag, Mo-

20   MLV Gag-RNase H, Mo-MLV Gag-SN, and Mo-MLV Gag-SN* were seen 10 to 14 days posttransfection, which coincided with maximum RCASBP(A) production. Immunoblot analysis indicated that the Mo-MLV Gag-RNase H polyprotein was expressed at a lower level than Mo-MLV Gag or Mo-MLV Gag-SN. Several viral proteins that were smaller than the expected full-length

25   polyproteins were also observed. These proteins were the likely result of partial degradation of the Pr85 or Pr65 polyprotein associated with the virion isolation procedure, since smaller Gag-associated proteins were also seen in the Mo-MLV Gag control. Southern blot analysis of genomic DNA isolated from day 24 cultures detected only intact proviruses. An *in situ* RNase H assay showed that

30   virus particles isolated from CEF expressing the Mo-MLV Gag-RNase H polyprotein contained high levels of RNase H activity. Little or no RNase H activity was detected in any other culture supernatants.

CEF Expressing the Mo-MLV Gag-RNase H Polyprotein Significantly

Inhibit Mo-MLV Spread. The antiviral effect of the CTVI fusions expressed by the CEF cultures was measured first in a prophylaxis assay. The cultures were challenged with a low dose (multiplicity of infection [MOI] of 0.05 to 0.1 focus-forming units [FFU] per cell) of amphotropic Mo-MLV strain Mo(4070A) (Ott et al., 1990; Ott et al., 1992). Mo(4070A) is an engineered hybrid Mo-MLV that contains the *env* gene and a portion of the *pol* gene of the amphotropic 4070A virus. The Mo(4070A) virus stock was produced on NIH 3T3 cells (1×10^6 to 2×10^6 FFU/ml). Infectious virus was quantitated by the S+L– focus assay on D56 cells (Bassin et al., 1971). The CEF cultures, which were dividing rapidly, were passaged for 14 days after Mo(4070A) infection. Infectious Mo(4070A) was quantitated at day 14 by the S+L– focus assay (Table 1). CEF expressing the Mo-MLV Gag-RNase H polyprotein reduced the level of infectious Mo(4070A) produced by more than 1,500-fold compared to the Mo(4070A)-infected CEF control.

Table 1. Inhibition of Mo-MLV spread by CTVI[a]

Construct used for treatment	Titer (FFU/ml)[b] (fold inhibition)[c]	
	Expt 1	Expt 2
None (mock treatment)	1.6×10^5	2.1×10^5
Vector alone	2.4×10^4 (6.6)	ND[d]
Mo-MLV Gag	2.5×10^4 (6.3)	4×10^4 (5)
Mo-MLV Gag-RNase H	1×10^2 (1,550)	9×10^1 (2,330)
Mo-MLV Gag-SN	5×10^1 (3,100)	1.6×10^1 (13,125)
Mo-MLV Gag-SN*	1.9×10^4 (8.4)	ND

[a] In a prophylaxis assay, CEF cultures seven passages after transfection with plasmids containing the RCASBP(A) retroviral vector-CTVI constructs were challenged with Mo(4070A) (multiplicity of infection, 0.05 to 0.1 FFU per cell). Infectious Mo(4070A) levels were quantitated from supernatants 14 days postinfection by S+L– focus assays. This experiment was performed twice.
[b] Titers were averages of duplicate assays.
[c] Fold inhibition was determined by comparing the Mo(4070A) titers of the experimental constructs to the Mo(4070A) titer on mock-treated CEF.
[d] ND, not done.

Several cultures were further characterized over the course of the 14-day
Mo(4070A) infection by quantitating the levels of infectious Mo(4070A) by
S+L- focus assay and analyzing the expression levels of the Mo-MLV Gag and
RCASBP(A) Gag polyproteins by Western immunoblotting. The amount of

5   infectious Mo(4070A) produced peaked 7 to 8 days after infection in the control
cultures (CEF alone and CEF expressing Mo-MLV Gag), and the titer remained
relatively constant over the next 6 days. In contrast, the amount of infectious
Mo(4070A) produced by CEF expressing either the Mo-MLV Gag-RNase H or
Mo-MLV Gag-SN polyprotein was significantly inhibited, although the levels of

10   infectious virus slowly increased over the course of the experiment. Mo(4070A)
virus production was also monitored by the appearance of the Mo-MLV CA
protein (about 30 kDa) on immunoblots. The levels of expression of the Mo-
MLV Gag, Mo-MLV Gag-RNase H, and Mo-MLV Gag-SN polyproteins
remained relatively constant throughout the experiment. At late time points

15   post-Mo(4070A) infection (days 12 to 14), the Mo-MLV Gag polyprotein levels
may decrease slightly due to proteolytic processing by protease from the
infectious Mo(4070A). The levels of the MA protein (about 19 kDa) from the
RCASBP(A) retroviral vector remained relatively constant throughout the
experiment.

20   The Mo-MLV Gag-SN fusion polyprotein was tested in parallel with the
Mo-MLV Gag-RNase H fusion to provide a direct comparison to a characterized
CTVI construct known to have antiviral activity. Unexpectedly, the antiviral
effect of Mo-MLV Gag-SN was 100-fold greater in the experiments reported
herein (> 3,000-fold [Table 1]) than that previously observed (about 30-fold

25   [Natsoulis et al., 1995]). The increase in the antiviral effect may be due to an
increase in the efficiency of producing CEF cultures that express higher levels of
the fusion polyprotein. The CEF themselves grew at a faster rate, presumably
due to changes in the growth media including different serum lots. This
difference in growth rate was especially apparent in CEF infected with

30   Mo(4070A). In previous experiments, Mo(4070A) infection dramatically
slowed the growth of CEF and caused some cytotoxicity.

       In the experiments reported herein, all CEF cultures grew at the same rate
until natural senescence. A consistent six- to eight-fold inhibition of Mo(4070A)

titer was observed in cells expressing the control constructs, including cultures
that expressed RCASBP(A) vector (Table 1), that was not detected previously
(Natsoulis et al., 1995; Schuman et al., 1996). Although this may be related to
the "steric effect" (three- to five-fold) of the Mo-MLV Gag-SN* polyprotein on

5   Mo(4070A) virus production observed by Schumann et al. (1996), the decrease
in the Mo(4070A) titers produced in cultures that expressed RCASBP(A)
suggests that the RCASBP vector may alter cells in ways that interfere with Mo-
MLV virus production, leading to a slight overestimate of the CTVI effect.
While several CTVI constructs have significant antiviral effects when used

10 prophylactically, the levels of infectious virus do increase during the experiment.
The production of a small number of viruses that do not contain CTVI-derived
polyproteins most likely accounts for the small number of infectious viruses
needed to spread the infection. Mutations in the *gag* gene capable of
circumventing this antiviral strategy would likely be exceedingly rare since they

15 would require the selection of Gag mutants that would still assemble normally
but be able to exclude Gag-degradative enzyme polyproteins from virions.

Delivery of the Mo-MLV Gag-RNase HI Fusion Polyprotein to Mo-
MLV-Infected CEF has a Therapeutic Effect. To assess the therapeutic potential
of the CTVI strategy, CEF chronically infected with Mo(4070A) were

20 transfected with the RCASBP(A) retroviral vector plasmids containing the Mo-
MLV Gag fusions and passaged for 16 days. For the therapy assay, CEF
chronically infected with Mo-MLV were generated by infecting 4×10^6 cells
with 2×10^6 FFU of Mo(4070A) (1 ml of virus stock) and passaging the culture
four to six times to allow maximum spread of the virus. The Mo(4070A)-

25 infected CEF cultures produced a maximum titer of 3×10^5 to 4×10^5 FFU/ml as
quantitated by the S+L- focus assay. The RCASBP virus spread through
Mo(4070A)-infected CEFs with kinetics similar to the spread on CEF that had
not been infected with Mo(4070A), reaching maximum levels after about 8 days
as determined by Western analysis of ALV MA levels. The levels of infectious

30 Mo(4070A) were quantitated throughout the experiment. The Mo(4070A) titers
at 14 days posttransfection in two experiments are shown in Table 2. The level
of infectious Mo(4070A) was reduced 7.5- to 18-fold in CEF expressing the Mo-
MLV Gag-RNase H polyprotein and 15- to 38-fold in CEF expressing the Mo-

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MLV Gag-SN polyprotein. Infection with the RCASBP(A) vector alone or

RCASBP(A) expressing the Mo-MLV Gag polyprotein lowered the titer of

Mo(4070A) produced by a chronically infected culture two- to three-fold

compared to Mo-MLV-infected CEF. This suggests that only a slight

5   therapeutic effect is directly attributable to the CTVI constructs once the

infection is established.

Table 2. Inhibition of Infectious Mo-MLV Produced after CTVI Treatment[a]

	Titer (FFU/ml)[b] (fold inhibition)[c]	
Construct used for treatment	Expt 1	Expt 2
None (mock treatment)	3×10^5	3.8×10^5
Vector alone	1.5×10^5 (2)	ND[d]
Mo-MLV Gag	1×10^5 (3)	1.6×10^5 (2.4)
Mo-MLV Gag-RNase H	4×10^4 (7.5)	2.1×10^4 (18)
Mo-MLV Gag-SN	2×10^4 (15)	1.3×10^4 (29)

[a] In a therapy assay, CEF chronically infected with Mo(4070A) were
transfected with plasmids containing the RCASBP(A) retroviral vector-CTVI
constructs. Levels of infectious Mo-MLV in supernatants were quantitated 14
20   days posttransfection by S+L– focus assays. The experiment was performed
twice.
[b] Titers were averages of duplicate assays.
[c] Fold inhibition was determined by comparing the mo-MLV tiers of the
experimental constructs to the Mo-MLV titer on mock-treated CEF.
25   [d] ND, not done.

Cleavage of the Mo-MLV Gag-Nuclease Fusion Junction by the

Incoming Mo-MLV Protease. An Mo-MLV Gag polyprotein fusion junction

30   was designed that could be cleaved by the protease of an incoming infectious

Mo-MLV by including the region six amino acids on either side of the normal

Mo-MLV protease cleavage site (PRCS). The new Gag fusion plasmid moves

the BamHI site to a position just downstream of the Gag termination codon

(pMGagPRCS). The termination codon was changed to a codon for glutamine,

35   the same amino acid that Mo-MLV inserts during suppression of the stop codon

when the Gag-Pol polyprotein is produced. Genes encoding the Mo-MLV Gag

PRCS-RNase H and Mo-MLV Gag PRCS-SN polyproteins were prepared to

determine if cleavage of the RNase HI or SN domain from the polyprotein would

increase the antiviral effect. A prophylaxis assay was used to measure

Mo(4070A) production in cells expressing the Mo-MLV Gag PRCS fusions and

the original Mo-MLV Gag fusions. Addition of the PRCS did not alter the level

of inhibition by the RNase HI or SN polyprotein, nor did inclusion of the PRCS

5   alter the therapeutic antiviral effect of either the Mo-MLV Gag-RNase H or the

SN polyprotein. Virion proteins isolated from day 16 of the therapy assay of

Mo-MLV-infected CEF and CEF expressing Mo-MLV Gag, Mo-MLV Gag-

RNase H, and Mo-MLV Gag PRCS-RNase H were assayed for RNase H activity

by *in situ* RNase H assay. Only virions from Mo-MLV Gag-RNase H and Mo-

10   MLV Gag PRCS-RNase H cultures contained measurable RNase H activity. A

new protein with RNase H activity about 22 kDa was observed in virions

obtained from cultures expressing the Mo-MLV Gag PRCS-RNase H

polyprotein. Presumably, this protein, which corresponds in size to *E. coli*

RNase HI, is the RNase HI domain cleaved from the Mo-MLV Gag PRCS-

15   RNase H polyprotein by the Mo-MLV protease. The ability to release a

degradative enzyme from the polyprotein should make it possible to design

future CTVI fusions in which activation of the degradative enzyme is controlled by protease cleavage (e.g., a zymogen), which would allow the use of degradative enzymes whose expression would otherwise be too toxic for the cell.

CTVI Inhibition of Mo-MLV Replication in Avian DF-1 Cells.   Line 0

5   CEF have been the cells of choice for the study of ALV, since line 0 does not contain endogenous loci related to ALV and produces high ALV titers. However, CEF are primary cells and have a limited life span (i.e., about 25 passages). Recently, a permanent cell line was derived from line 0 CEF, named DF-1 (Dr. Douglas Foster, University of Minnesota). Previously, permanent

10   avian cell lines have been unsatisfactory for many ALV studies due to the poor growth of ALV or the presence of interfering exogenous and/or endogenous viruses. The growth of ALV-based retroviral vectors and Mo-MLV was compared in DF-1 and CEF. The DF-1 and CEF cells grow at similar rates and support vigorous growth of ALV and Mo-MLV. The rate at which both ALV

15   and Mo-MLV reach maximum titers, and the level of viral protein produced, is approximately the same in either cell type. The titer of the ALV-based retroviral vectors is 5-10-fold higher when produced in DF-1 cells compared to CEF. A slight increase in the titer of Mo(4070A) viral stocks was obtained when grown in DF-1 cells compared to CEF. These data demonstrate that the DF-1

20   permanent cell line can be used to test antiviral strategies *in vitro*, and also to generate clonal cell lines that support vigorous ALV replication. Table 3 summarizes the CTVI antiviral effects on Mo-MLV replication in DF-1 cells both prophylaxis and therapy assays.

In animal model systems, it is possible to deliver genes by using either

25   DNA microinjection or retroviral infection into an embryo. This eliminates the host's immune response, and such methods can be used to deliver genes efficiently. Thus, the CTVI strategy may be useful *in vivo*, e.g., in animal systems in which viral infections have important economic consequences (e.g., agriculture), or in non-human transplant tissues.

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Table 3. CTVI inhibition of Mo-MLV replication in avian DF-1 cells

| Construct | Titer (FFU/ml)[a](fold inhibition)[b] | | |
| | Prophylaxis Assay[c] | Therapy Assay[d] | |
		1st Delivery[e]	2nd Delivery[f]
None (mock)	3×10^5	2×10^5	2.1×10^5
Mo-MLV Gag	1.2×10^5 (2.5)	1.4×10^5 (1.4)	1.4×10^5 (1.5)
Mo-MLV Gag-RH	<10 (>30,000)	1×10^2 (2,000)	<10 (>21,000)
Mo-MLV Gag-SN	<10 (>30,000)	1×10^2 (2,000)	<10 (>21,000)

[a] Titers were averages of duplicate assays.

[b] Fold inhibition was determined by comparing the Mo(4070A) titers of the construct to mock-treated DF-1 cells.

[c] DF-1 cells chronically infected with RCASBP(A)-CTVI construct were challenged with Mo(4070A) (multiplicity of infection, 0.05-0.1 FFU per cell). Infectious Mo(4070A) levels were quantitated from supernatants by S+L- assay 30 days post-infection.

[d] DF-1 cells chronically infected with Mo(4070A) were infected with RACSBP(A)-CTVI or RCASBP(C)-CTVI construct viruses. The levels of infectious Mo(4070A) in supernatants were quantitated by S+L- assay 30 days post-infection.

[e] RCASBP(A)-CTVI viruses were used to initiate the therapy assay with the maximum antiviral effect achieved at 30 days.

[f] The antiviral constructs were delivered a second time by the RCASBP(C) vectors.

Example 2

Human Tropic PERVs Produced by Pig Primary PBMC

Methods

Cells and Cell Culture. The cell lines used were either obtained from the ATCC unless otherwise indicated. 293 human embryonic kidney (ATCC CRL-1573), MMK *Mus musculus molossinus* kidney (ATCC CRL-6439), SC-1 mouse embryo epithelial cells (ATCC CRL-6450), ST-IOWA (pig fetal testis), 1o PT (pig testis), MDTF *Mus dunni* tail fibroblast (kindly provided by Olivier Danos), NRK normal rat kidney cells (ATCC CRL-6509), Rat2 rat embryo fibroblasts (ATCC CRL-1764), SIRC rabbit corneal fibroblast (ATCC CCL-60), D17

canine osteosarcoma (ATCC CRL-6248), MDBK bovine kidney epithelium

(ATCC CCL-22), FRHK4 rhesus monkey kidney cells (ATCC CRL-1688),

Mv1Lu mink lung epithelial cells (ATCC CCL-64), MiCL.1 (S+L-) mink lung

(ATCC CCL-64.1), Fc3TG feline tongue fibroblast (ATCC CCL 176), AK-D

5   feline lung epithelial cells (ATCC CCL-150), CRFK feline kidney epithelial

cells (ATCC CCL-94), CaKi-1 clear cell kidney carcinoma (ATCC HTB-46),

HeLa cervical adenocarcinoma (ATCC CCL-2), HOS human osteosarcoma

(ATCC CRL-1543), CaCo-2 colorectal adenocarcinoma (ATCC HTB-37) were

maintained in Dulbecco's modified Eagle's medium supplemented with 10%

10   fetal bovine serum (FBS), 2 mM glutamine, 1 mM sodium pyruvate, 100 U of

penicillin per ml, and 100 μg of streptomycin per ml.   PG-4 feline

glial and astrocytes (ATCC CRL 2032) were maintained in McCoy's 5A

medium supplemented with 15% FBS, 2 mM glutamine, 1 mM sodium pyruvate,

100 U of penicillin per ml, and 100 μg of streptomycin per ml.   HepG-2

15   hepatoblastoma (ATCC HB-8065) and HT1080 fibrosarcoma (ATCC CCL-121)

were maintained in Eagle's Modified Essential Medium supplemented in a

similar manner. The following cell lines were maintained in RPMI 1640

supplemented with 10% FBS, 2 mM glutamine, 0.05 mg gentamicin sulfate per

ml, 1X non-essential amino acids (Biofluids, Rockville, MD): Molt 4 acute

20   lymphoblastic (ATCC CRL-1582), Daudi Burkitt's lymphoma (ATCC CCL-

213), Raji Burkitts lymphoma (ATCC CCL-86), U937 histiocytic lymphoma

(ATCC CRL 1593.2) UCLA-SO-M14 T cells (Ramsdell et al., 1988), and the

human natural killer cell line YTN10 (Yodoi et al., 1985).   PBMCs were

obtained from NIH and Yucatan minipigs.

25   Primary pig aortic endothelial cells are isolated by incubating the inner

layer of pig aortas with 0.2% collagenase type I for 20 minutes at 37°C.

Detached cells are washed with phosphate-buffered saline, and cultured in M199

medium supplemented with 10% fetal bovine serum, 25 mM HEPES, 2 mM

glutamine, 100 U per ml of penicillin, and 100 μg streptomycin. Cultures are

30   fed three times per week and subpassaged every 4-7 days.

               Pig hepatocytes are isolated by a two-step perfusion technique as

described by Sielaff et al. (1995). The liver is treated by systemic heparinization

before isolation. The liver is then treated with an EDTA and collagenase D

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solution to free the hepatocytes. The hepatocytes are isolated by gently combing

the liver, washing the dislodged cells in Williams E medium supplemented with

5% calf serum, 2 mM L-glutamine, 15 mM HEPES, 1.5 mg/ml insulin, 100 U

penicillin per ml and 100 µg streptomycin per ml. The freshly harvested

5   hepatocytes are cultured in collagen gel.

       Derivation of viral pseudotypes. Cells productively infected with wild-

type PERV were superinfected with retroviral vector-containing supernatant

harvested form PA317/G1BgSvN (McLaughlin et al., 1993), in a manner similar

to that previously described (Leverett et al., 1998), to generate PERV

10   pseudotypes carrying the murine retrovirus genome coated by PERV core and

envelope proteins. G1BgSvN-containing cells were selected in 187 µg/ml G418

(active component). After 10-14 days selection, G418-resistant colonies were

pooled and used to generate viral pseudotypes. Since the G1BgSvN vector

genome contains the coding sequence for both neomycin phosphotransferase and

15   for β-galactosidase, pseudotype infection is monitored by the acquisition of

target cell resistance to G418 or by immunohistochemical detection of target

cells expressing β-galactosidase.

       Infectivity assays. Supernatant infections were used to determine the

infectivity of cell-free virus derived from different producer cell lines.

20   Comparisons between fresh and frozen virus-containing supernatants

demonstrated that storage of viral supernatants at -70°C resulted in > 100-fold

decrease in infectious titer. Therefore, all experiments are performed using

freshly harvested supernatant. On the day prior to initiating an infectivity assay,

target cells were seeded at 3-5 x 10^4 cells per well in 12-well dishes. On the next

25   day, supernatant from post-confluent cultures of virus producer cells was

harvested, filtered through a 0.45 µm filter, adjusted to 6 µg/ml polybrene and

used to replace the medium on target cells. On the following day, the cells were

fed with fresh medium. When β-galactosidase expression was used to monitor

infectivity, the cells were fixed and histochemically stained 48-72 hours post-

30   exposure to viral supernatant and observed microscopically to enumerate blue-

staining foci of cells (BFU/ml), as described elsewhere (Wilson et al., 1991). If

the experiment was performed to assess productive infection of the target cell,

~~the cells would be passaged 1-2 times per week as needed during the course of~~
the experiment and monitored as described below.

The coculture of target cells with virus-producer cells was used to assess
the ability of the virus to be transmitted to non-adherent cells. Since several

5   different cell types were used as a source of virus-producer cells in these
experiments, the dosage of irradiation required to ensure the virus-producer cells
would die within 5 days after irradiation was first determined. Three to four x
10^6 cells for each virus-producer cell line were subjected to 2,000,5,000 or
10,000 rads (using a ^{137}Cs source, Nordion GammaCell 100) and seeded into a

10   96-well plate at 2-3 x 10^5 cells/well. Irradiated and control non-irradiated cells
were monitored for proliferation daily for 5 days by measuring [^{3}H]TTP uptake
after cells were cultured for 8 h with 1.0 μCi [^{3}H]TTP (6.7 Ci/mmol; Dupont
NEN, Boston, MA), harvested (Skatron, Sterling, VA) and processed for
scintillation counting. Based on these findings, the following conditions were

15   used: Daudi cells received 2000 rad, Molt4 and U937 received 5000 rads.

When the target cells in the coculture were human peripheral blood
mononuclear cells (hPBMC), the mononuclear layer was collected from a buffy
coat from a human donor separated by lymphocyte separation medium (Organon
Teknika, Durham, NC). Cells were stimulated in 1X PHA (according to

20   manufacturer's instructions, Life Technologies, Gaithersburg, MD) for 3 days
and then mixed in a 1:1 ratio with 3 x 10^6 lethally irradiated virus-producer cells
in 12-well dishes. Thereafter, the cocultures were maintained in RPMI 1640
medium supplemented with 10% FBS, 10% purified human IL-2 (Boerhinger
Manheim), 10 ng/ml recombinant human IL-2 (Chiron Corporation, Emeryville,

25   CA) 2 mM glutamine, 100 U penicillin, and 100 μg per ml of streptomycin.
Control wells containing irradiated virus-producer cells only were monitored for
cell viability by visualizing samples microscopically for trypan blue exclusion.
Cocultures containing 293 cells as target cells were carried out in parallel as a
control for the infectivity of the virus-producer cells. After initiation of the

30   coculture, the next day the medium was replaced with fresh medium and cells
were sub-cultured 1-2 times/week as needed. Cocultures containing the hPBMC
as targets were reinfused every two weeks with freshly-stimulated hPBMC from
the same buffy coat of cells. Methods to monitor virus infection of target cells.

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~~Infection of target cells was assessed by one of three different methods.~~ In some
cases, the titer of infectious virus pseudotypes was determined by β-
galactosidase expression. Cells were histochemically stained 48-72 hours after
exposure to virus-containing supernatant, and foci of β-galactosidase expressing

5   blue cells were counted (BFU/ml). Those titers were then normalized to the titer
observed on 293 cells exposed to the same viral supernatant and are reported as
% of control. The titer observed on 293 cells ranged 10-fold from several
hundred BFU/ml to 2-3,000 BFU/ml.

Virion isolation and purification. Culture supernatants are harvested

10   from infected cells, cleared of floating cells and debris by centrifugation at 3000
× g for 10 minutes, and the virus stocks stored at -80°C. Virions are isolated for
RT assays and Western analysis by ultracentrifugation of 10 ml virus stock
underlaid with 1 ml 20% sucrose at 35,000 rpm in a Beckman SW40 rotor for 1
hour at 4°C to pellet the virus (Federspiel et al., 1994). The viral pellet is then

15   resuspended in either RT assay buffer or Laemmli load buffer.

PERV virions are further purified for antibody production either by
sucrose equilibrium density centrifugation or by a sucrose step gradient to
concentrate the virions and remove proteins, nucleic acids and ribosomes from
the cell supernatants. For equilibrium gradients, the supernatants are mixed with

20   sucrose to a density slightly higher than 1.16 g/ml, the average buoyant density
of retroviral particles, and the gradient formed by ultracentrifugation. Viral
supernatants are layered onto a two-step sucrose gradient, 20% and 50%, and the
virions banded at the boundary between the sucrose steps. In both methods, the
banded virions are harvested by removing the band with a syringe through the

25   side of the tube to prevent contamination.

PCR. Standard PCR reactions contains 1.25 µl of 10X PCR buffer (50
mM Tris, pH 8.3, 50 mM KCl, 7 mM MgCl$_2$), 1.25 µl of 1.7 mg/ml BSA, 0.5 µl
of each dNTP at 25 mM, 0.56 µl of each oligonucleotide primer (A_{260} = 5), 0-8%
dimethyl sulfoxide, 4.2 µl water, and 1 µl genomic DNA (about 100 µg/ml)

30   (Federspiel et al., 1996). The reactions are heated to 90°C and initiated with 1.5
µl *Taq* DNA polymerase (0.75 units). Thirty cycles of PCR are carried out as
follows: 90°C for 20 seconds, then 59°C for 80 seconds. Alternations of this
basic protocol follow the recommendations of the polymerase manufacturer.

Three sets of primers specifically amplify the three PERV envelope

subgroups in the SU glycoprotein region: (all primers listed 5'-3') PERV-A:

TGGAAAGATTGGCAACAGCG and AGTGATGTTAGGCTCAGTGG (SEQ

ID NO:8 and SEQ ID NO:9, respectively); PERV-B:

5   TTCTCCTTTGTCAATTCCGG and TACTTTATCGGGTCCCACTG (SEQ ID

NO:10 and SEQ ID NO:11, respectively); PERV-C:

CTGACCTGGATTAGAACTGG and ATGTTAGAGGATGGTCCTGG (SEQ

ID NO:12 and SEQ ID NO:13, respectively). PCR products were separated on a

1.0% agarose gel and DNA fragments of 2-2.5 kB were purified using Qiaex gel

10   purification kit (Qiagen, Valencia, CA). Purified PCR products were then

cloned into the TA vector using the TA Cloning Kit (In Vitrogen, Carlsbad, CA).

Restriction analysis of clones derived from the TA vector was used to ensure an

insert of the appropriate size was present. A minimum of 4 representative clones

was chosen for sequence analysis. All deoxynucleotide sequencing was

15   performed by the Mayo Clinic Molecular Biology Core on a Perkin Elmer

ABIPRISM™ 377 DNA Sequencer (with XL Upgrade) with the PE applied

Biosystems ABI PRISM™ dRhodamine Terminator Cycle Sequencing Ready

Reaction Kit with AmpliTaq DNA Polymerase (PE Applied Biosystems, Foster

City, CA). The ClustalW Multiple Sequence alignment Program of Mac Vector

20   6.0.1 (Oxford molecular Group) was used to compare the deduced envelope

amino acid sequences.

RT-PCR. RNA is isolated from virions with RNA STAT 50-LS or

STAT-60 (Tel-Test, Inc., Friendswood, TX), and converted to cDNA with 2.5

µM random hexanucleotide primers and Superscript II (Life Technologies,

25   Gaithersburg, MD) (Wilson et al., 1998). cDNA templates are amplified with

primers corresponding to the PERV *pol/env* gene boundary

(ACCTCGAGACTCGGTGGAAGGG; SEQ ID NO:14) and the untranslated

region 3' of the PERV *env* gene (CTGGGTTCTGGGAGGGTTAGGTTG; SEQ

ID NO:15), or amplified with PB906 (5'

30   ACGTACTGGAGGAGGGTCACCTGA 3'; SEQ ID NO:16) and PB908 ('5

GTCCCGAACCCTTATAACCTCTTG 3'; SEQ ID NO:17) or PERVenv1

(sense) (5' ACCTCGAGACTCGGTGGAG; SEQ ID NO:11) and PERVenv2

(anti-sense) (5' CTGGGTTCTGGGAGGGTTAGGTTG; SEQ ID NO:12) for

30 cycles at 94°C for 30 seconds, at 60°C for 30 seconds, and at 72°C for 1

minute. The amplified products are cloned into the PCRII T-A vector
(Invitrogen Corp., San Diego, CA).

RT assays. The RT assays are conducted as described in Wilson et al.
(1994) and Wilson et al. (1998) and are optimized for the detection of PERV RT
as reported by Phan-Thanh et al. (1992). Pelleted virions or virions in cleared
supernatant are treated in solubilization buffer (25 mM NaCl, 0.20% Triton X-
100, 10 mM Tris, pH 7.5) for 15 minutes at ambient temperature. The
solubilized samples are incubated for 3 hours at 37°C in 2X substrate buffer (50
mM Tris, pH 7.5, 10 mM dithiothreitol, 0.6 mM $MnCl_2$, 10 μg of poly (rA)-poly
$(dT)_{12-18}$ (Pharmacia, Piscataway, NJ) per ml, and 10 μCi of ^{3}H[TTP] per 25 μl
(22 Ci/mmol). The incubation is terminated with EDTA followed by blotting
the synthesized DNA on a DEAE filtermat (LKB-Wallac, Gaithersburg, MD)
through use of a harvesting apparatus (Skatron, Sterling, VA) with a rinse of 1%
Na_2HPO_4 (pH 7.0) containing 1 mM EDTA. Radioactivity is then determined
via liquid scintillation. All samples are assayed in triplicate.

RNase protection assays (RPA). RNA from viral particles or cells is
extracted using RNAzol™ B (Tel-Test, Inc., Friendswood, TX). RPA are done
with the RPA II Ribonuclease Protection Assay Kit (Ambion, Inc., Austin, TX).
Antisense riboprobes are synthesized in the presence ^{32}P[UTP] using the RNA
Transcription Kit (Stratagene, La Jolla, CA). In standard RPA conditions,
labeled riboprobe (5×10^5 cpm) is incubated with the RNA at 50°C overnight.
The annealed reaction is digested with an RNase A/T1 mix at 30°C for
45 minutes. The samples are cleaned up and fractionated on 6% acrylamide-7.6
M urea gels, dried and exposed to Kodak X-OMAT film.

Western analysis. Virion proteins were isolated and separated on 12%
SDS polyacrylamide gels. The proteins were transferred to nitrocellulose filters
with an Enprotech blotting apparatus (Integrated Separation Systems, Hyde Park,
MA). The filters were blocked in phosphate-buffered saline with 10% nonfat dry
milk (wt/vol) for 1 hour at ambient temperature. The blocked filters were
drained and incubated with a 1:1000 of goat anti-GALV CA or goat anti-SSAV
CA antisera in rinse buffer (100 mM NaCl, 10 mM Tris, pH 8, 1 mM EDTA,
0.1% Tween 20) containing 1% nonfat dry milk for 1 hour at ambient

temperature (Biological Carcinogenesis Branch Repository of the National

Cancer Institute). The filters were then washed extensively in rinse buffer, and

incubated with 50 ng/ml peroxidase-labeled rabbit anti-goat IgG (H + L)

(Kirkegaard and Perry, Gaithersburg, MD) in rinse buffer with 1% nonfat dry

5   milk for 1 hour at ambient temperature. The filters were washed and the protein-

antibody-peroxidase complexes detected with the Western Blot

Chemiluminescence Reagent (DuPont NEN, Boston, MA). The filters were

exposed to Kodak X-OMAT film.

Results

10   Host Range of PERV Isolates

Primary PBMC were isolated from an NIH miniature pig and exposed to

two different combinations of mitogens: phytohemagglutinin (PHA) and

phorbol myristate acetate (PMA) or PMA and the calcium ionophore A23187.

Mitogenic activation by either treatment resulted in a sharp increase in reverse

15   transcriptase (RT) activity levels five days after stimulation (Figure 1A). To

determine if the increase in RT activity correlated with the production of

infectious virus, stimulated PBMC from NIH and Yucatan minipigs were

cocultured with the pig ST-IOWA cell line, a line that does not produce

significant endogenous RT activity levels in the supernatants but is susceptible to

20   PERV infection. Both cultures produced detectable RT activity 14 days after

exposure to activated PBMC (Figure 1B). These results indicated that mitogenic

activation of the pig PMBC released PERV capable of productive infection of

pig cells. To determine if the produced PERV can infect human cells, stimulated

PBMCs were cocultured with the human embryonic kidney 293 cell line. RT

25   activity was detected in 293 cells at day 31 (Figure 1C) and continued to increase

to levels comparable to RT activity levels produced by infected ST-IOWA cells.

These results clearly demonstrated that the stimulated pig PBMC produce PERV

that can productively infect human cells. These findings suggest that the

stimulation of porcine lymphocytes, transferred to the patient in a xenograft, may

30   result in the production of replication-competent human-tropic PERV capable of

infecting the patient.

In order to test if the PERVs of this primary 293 infection (1°-

293/PERV) could improve their infection efficiency in human cells, supernatant

from the 1°-293/PERV culture was passaged into a new culture of human 293

cells (2°-293/PERV) and allowed to spread through the culture (Figure 2). After

the culture reached maximum RT activity, supernatant from the 2°-293/PERV

culture was passaged into fresh 293 cells and allowed to spread (3°-293/PERV).

5   The titer of the PERVs produced by these three cultures was quantitated by

generating MLV-βgal pseudotypes.

The titers of all three PERV cultures were approximately the same on the

control ST-IOWA cells (Figure 3). However, the titers of the MLV-βgal/PERV

pseudotypes produced from the 2°-293 and 3°-293 cultures were 5-fold higher

10   compared to the 1°-293 culture. The relative copy number of integrated PERV

proviruses in these three PERV infected 293 cultures was quantitated by

Southern analysis. As shown in Figure 4, the DNA isolated from the 2°-

293/PERV and 3°-293/PERV cultures contained more integrated PERV

proviruses per cell relative to the DNA of the 1°-293/PERV cultures.

15   Overall, the host range of the PERVs produced from the 293/PERV

cultures appears to coincide with a PERV-A related isolate (see Table 4

compared to Takeuchi et al., 1998). One notable exception is the inability of

PERV-1.15 MLV-βgal pseudotypes to infect D17 cells, suggesting that the

changes in the envelope glycoproteins may have altered the host range.

Table 4. Host range of PBMC PERV produced by 293 cells

Species	Cell	Titer of *LacZ* pseudotype[a]		
		1°	2°	3°
Human	HeLa	+	+	+
	HOS	+	+	+
	Caki-1	–	–	+
	293	+	++	++
Primate	FRhK-4	–	–	–
Pig	ST-IOWA	++	++	++
	1o PT	+	+	+
Dog	D17	–	–	–
Rabbit	SIRC	–	–	–
Hamster	E36	–	–	–
Mink	Mv-1-Lu	++	++	++
	MiCl1	+	++	++
Cat	Fc3Tg	+	+	++
	AK-D	+	+	++
	CRFK	++	++	++
	PG4	++	++	++

[a] MLV-βgal pseudotypes were produced in the PERV infected human 293 cultures: negative (–); 1-100 BFU/ml (+); >100 BFU/ml (++).

Table 5. Productive infection of human and animal cell lines by 293/PERV

Cell Line	Virus[a]	*LacZ*[b]	RT[c]	RT-PCR[d]
ST	1°	++	neg	nd
(pig testis)	2°	++	d.9	nd
	3°	++	d.9	nd
293	1°	+	d.18	nd
(human kidney)	2°	++	d.10	nd
	3°	++	d.10	nd
Caco-2	1°	?	neg	–
(human colon)	2°	?	neg	–
	3°	?	d.18	–
HepG2	1°	++	neg	–
(human liver)	2°	++	d.26	+
	3°	++	d.26	+
HT1080	1°	+	neg	–
(human fibrosarcoma)	2°	++	d.26	+
	3°	++	d.13	+
Mv-1-Lu	1°	+	neg	+
(mink lung)	2°	++	d.21	+
	3°	++	d.38	+
CRFK	1°	+	neg	+
(cat kidney)	2°	+	d.14	+
	3°	++	d.14	+

[a] Culture supernatant from 1°, 2°, or 3° -293/PERV cells.

[b] Titer of MLV-βgal pseudotype: negative (–); 1-100 BFU/ml (+); >100 BFU/ml (++).

[c] The day when the RT activity was greater than 2-fold over background activity from uninfected cells. The assay was terminated after 8 weeks.

[d] At 8 weeks postinfection, RNA was isolated from the culture supernatant and assayed for *pol* sequences by RT-PCR.

nd Not determined.

? High background staining made the data uninterpretable.

PERV Produced by the 293/PERV Cultures Can Productively Infect Other Human, Mink and Cat Cell Lines. A number of different cell lines were employed to determine the tissue and species specificity of PERV produced by 293/PERV cultures (Table 5). All the cell lines tested except Caco-2 were productively infected by the virus populations produced by the 2° and 3°-- 293/PERV cultures. The 3°-293/PERV infected Caco-2 culture produced RT

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activity at day 18 and detectable *pol* RNA by RT-PCR indicating a productive

infection. The 1°-293/PERV culture produces 5-fold lower PERV titers (see

Figure 3) which may account for the lower replication efficiency in most lines.

The transfer of significant levels of replication-defective PERV genomes into the

5   primary 293 cells may account for the lower titers. The ability of the PERV

produced by the 2° and 3°-293/PERV cultures to efficiently replicate in most

cultures may be the result of the loss of defective interfering PERV genomes or

the adaptation of the virus, or both.

An expanded analysis of species tropism indicated that cell lines derived

10 from mouse, rat, rabbit, dog, cow, and rhesus monkey were resistant to infection

by 293/2° pseudotypes, while cell lines from cat or mink cell lines were

susceptible. This latter susceptibility was low compared to 293 cells, with the

exception of MiCL and PG4 cell lines. Mink and cat cell lines were also

infected with PERV infected 293/2° supernatant. Only low levels of RT activity

15 were observed in MiCL and CRFK cultures. PG-4 cultures had RT activity of

933 cpm [^{3}H-TTP] at 3 weeks, which was reduced to 2344 cpm by 5 weeks, post

infection.

Analysis of the Susceptibility of Hematopoietic Cells to Infection by

PERV. In order to assess whether primary human PBMC (hPBMC) were

20 susceptible to infection by PERV, PHA-activated hPBMC were cocultured with

irradiated 293/2° virus-producer cells and maintained in IL-2-containing medium

for 8 weeks. No RT activity above background levels or viral RNA as measured

by RT-PCR were produced during the course of the experiment. Since

maintenance of the hPBMC in IL-2 during the course of the experiment biases

25 the culture conditions towards the proliferation of T cells, other hematopoietic

lineages that may be susceptible to infection may not have been represented. To

investigate the possibility that other hematopoietic lineages may be permissive

for infection, a number of human hematopoietic cell lines representing the T cell,

B cell, myeloid, and NK cell lineages were analyzed for their susceptibility to

30 PERV infection by coculture with irradiated 293/2° virus producer cells.

Supernatants from the cocultures containing either M14 (T cell lineage), Jurkat

(T cell lineage), K562 (myeloid lineage), or the NK cell line YTN10, remained

negative for RT activity during the 8 week course of the experiment, although

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viral RNA could be detected by RT-PCR throughout the experiment. Figure 5
shows the data from the RT activity for the cocultures containing the T cell line
Molt 4, the B cell lines Daudi and Raji, and the myeloid cell line U937. By 4
weeks post-coculture, the RT activity in the Molt 4- and Daudi-containing

5   cocultures was positive and continued progressively to increase throughout the
period of the coculture. In contrast, the RT activity for both the Raji and U937
sets of cocultures plateaued by 4 weeks post-culture. The results from this
experiment suggest that the Molt 4, Daudi, Raji, and U937 cell lines were
permissive for productive infection by PERV.

10   The RT positive hematopoietic cell lines were then co-cultured with
primary hPBMC, as a virus population may have been selected in the susceptible
hematopoietic cell lines that could more efficiently infect primary hematopoietic
cells. The irradiation conditions optimal for lethal irradiation were determined
for each of the RT positive Daudi, Molt 4 and U937 cell lines. Then lethally-

15   irradiated RT-positive Daudi, Molt 4 and U937 were each employed as virus-
producer cells in cocultures with primary hPBMC activated with PHA or with
human 293 cells as positive controls. By 2 weeks post-coculture, each of the
cocultures containing 293 cells as target cells became significantly RT positive
(>7,000 cpm), and by 3 weeks, the RT activity in each of these cultures

20   increased to >20,000 cpm. By contrast, none of the cocultures containing the
hPBMC demonstrated RT activity higher than negative control cultures over the
course of the experiment. Although viral RNA was detected by RT-PCR in the
supernatant of cultures sampled 1-2 weeks post-exposure, the supernatant was
negative for viral RNA by RT-PCR by three weeks and remained so out to the 8-

25   week time point. The positive RT-PCR results obtained at the early time points
most likely reflect presence of residual irradiated virus-producer cells which
disappear from the culture at the later time points, rather than infected cells.

Table 6. Comparison of the nucleotide and deduced amino acid sequence
of PERV-1.15 with the three known PERV *env* subgroups.

	1.15	A	B	C
PERV-1.15	–	96	85	73
PERV-A	97	–	82	76
PERV-B	87	84	–	70
PERV-C	72	76	72	–

Amino Acid Identity (%)

Nucleotide Identity (%)

Env Subgroups of PERV in PERV- infected 293 Cultures

To determine what PERV envelope subgroups were present in virions
produced by the 293/PERV populations, the envelope regions was cloned by
RT-PCR amplification of virion RNA isolated from 1°, 2°, and 3°-293/PERV
supernatants. The primers used for RT-PCR were designed from the PERV-A
and PERV-B envelope sequences. Oligonucleotide primers were synthesized
homologous to a conserved region at the 3'-end of the *pol* gene (5' of the PERV
env gene) and a conserved untranslated region 3' of the *env* gene. The amplified
products were cloned into the pCRII T-A cloning vector (Invitrogen) and
representative clones sequenced from each culture. Unexpectedly, only one
PERV envelope gene species was detected in virions produced from all three
cultures. The deduced amino acid sequence shows that the surface glycoprotein
region of the gene is almost identical to PERV-A, but the transmembrane
glycoprotein region of the gene is almost identical to PERV-C (Figure 6). The
nucleotide and amino acid sequence homologies of PERV-1.15 with PERV-A, -
B, and -C are summarized in Table 6.

While the PERV-1.15 envelope gene is clearly in the PERV-A receptor
subgroup family, the gene contains a unique transmembrane glycoprotein that
may be important for efficient PERV replication in human cells. The variation
in the PERV *env* genes may be significant since variant MLV *env* genes have

been shown to not only broaden the host range of the esotropic MLV but

increase the pathogenicity (Rosenberg et al., 1997). These viruses arise by

recombination between different endogenous MLVs. Recombination between

env sequences of exogenous and endogenous feline leukemia viruses (FeLV)

5   also occurs in FeLV-induced thymic tumors.

All 293/PERV cultures contained PERV-A envelope sequences as

determined by PCR using subgroup-specific envelope primers. However, PERV-

B envelope sequences were not detected in the 293/PERV culture DNAs. The

PERV-C envelope sequence was detected in the 1°-293/PERV culture, but not in

10   the subsequent virus passages. PERV-C does not infect most human cells

efficiently.

Isolation of Other Human-Tropic PERVs. To identify other human-

tropic PERV, primary cell cultures, e.g., pig aortic endothelial cells (PAEC from,

for example, NIH and Yucatan minipig breeds), pig PBMC, and primary pig

15   hepatocytes, from normal pig tissues are assayed for the production of human-

tropic PERVs on human 293 cells. The cultures are passaged and monitored for

PERV production by RT-PCR and RT activity. The PERV populations are

passaged onto fresh human cells to generate secondary cultures to determine if a

particular PERV species dominates, and if the overall replication efficiency

20   improves. Env subgroups and LTR-U3 sequences are determined as described

above.

New preparations of PERV cDNA are synthesized by RT-PCR from

virion RNA isolated from PERV sources, and 1°, 2°, and 3°-293/PERV culture

supernatants. The region from the virion mRNA polyA tail to the PERV *pol*

25   gene is amplified by RT-PCR. The RT-PCR reactions use a *pol* primer that

hybridizes just 5' of the *env* gene, and a second reaction using a *pol* primer

homologous to the RT active site region. Two reactions that use different *pol*

primers increase the chance of amplifying all PERV variants. ProSTAR Ultra

High Fidelity (HF) RT-PCR System (Stratagene) is employed for RT-PCR as it

30   combines high fidelity with long target amplification using the *PfuTurbo* DNA

polymerase. *PfuTurbo* DNA polymerase efficiently generates blunt ends. The

product is cloned into PCR-Script (Stratagene) designed for blunt-end cloning.

This approach does not rely on specific restriction sites to be present in the

PERV sequence, and produces cDNA clones that contain both the envelope gene and the U3 LTR region.

The resulting bacterial colonies are grouped first by envelope subgroups by probing colony lifts on nitrocellulose with ^{32}P-labeled DNA probes specific

5   for each PERV envelope subgroup. These regions only detect the variable region of the envelope surface glycoprotein. The probing colony lifts with a specific ^{32}P-labeled probe (DNA region or oligonucleotide), by PCR with specific primers designed to identify the variant sequence, and/or by Southern blots of digested plasmid clone DNA probed with a specific ^{32}P-labeled probe.

10   In this way, thousands of colonies are easily probed for specific PERV sequences, thereby increasing the detection of rare PERV RNAs. For example, PERV-B was not detected in the DNA in any of the 293/PERV cultures, and PERV-C was barely detected in the DNA of 1°-293/PERV. Thus, PERV-B and PERV-C envelope sequences may be represented in virions, especially in virions

15   produced by PBMC and the 1°-293/PERV culture supernatants. RT-PCR reactions designed to amplify only PERV-B and PERV-C mRNAs are also done. Finally, the expression profile of PERV envelope and U3-LTR sequences in PBMC is analyzed by RNAse protection assays. Specific PERV envelope and U3-LTR sequences are cloned into pBluescript KS (Stratagene), and used to

20   produce ^{32}P-labeled antisense RNA probes. Pig and human GPDH probes are also used to normalize RNA levels in the assays for quantitation of PERV RNA levels in PBMC. These assays determine if PERV-B and PERV-C loci are expressed in PBMC. The nucleotide sequence is then determined for 10-15 representative clones of each unique PERV group.

25   Cellular DNA is probed, by Southern analysis and PCR, for the presence of any new or variant PERV sequence identified by these experiments. This analysis verifies that the PERV sequence is present in the genome, and not an artifact of the cloning or analysis procedure, although the sequence could be the result of adaptation. The biological properties of all PERV sequences is

30   characterized in an infectious molecular clone. RNase protection assays are used to identify and quantitate the expression levels of the unique PERV elements in RNA isolated from a variety of tissues: brain, pancreas, spleen, liver, heart, kidney, muscle, lung, and PBMC.

Example 3

Molecular Cloning and Characterization of PERVs

A lambda genomic library was prepared from *Sau*3A-partially digested DNA isolated from 2°-293/PERV cells in the Lambda Dash II vector

5 (Stratagene). The library was probed with a ^{32}P-labeled PERV *pol* gene fragment, and ten genomic clones isolated. Six of the clones were unique and contained partial PERV proviruses.

All of the cloned PERV regions were nearly identical, with at most two amino acid changes in the coding regions, except the PERV sequences in lamA8

10 (Figure 7). The lamA8 *pol* gene contains an 86 nucleotide deletion that results in the truncation of the open reading frame at amino acid 887, a loss of 308 amino acids. The lamA8 *env* gene contains 101 nucleotide insertion that results in the truncation of the open reading frame at amino acid 599, a loss of 60 amino acids which includes the putative transmembrane region of the transmembrane

15 glycoprotein. It is unlikely, therefore, that lamA8 contains a functional *pol* or *env* gene.

The PERV regions cloned from the 2°-293/PERV culture were compared to the putative complete PERV cDNA, PERV-MSL (Akiyoshi et al., 1998), the PERV-A (Letissier et al., 1997) and PERV-1.15 envelope genes, and gibbon ape

20 leukemia virus (GALV) (Table 7). The 293/PERV *gag* and *pol* genes, as well as the 5′ and 3′ untranslated regions were nearly identical to the PERV-MSL nucleotide and deduced amino acid sequence.

Table 7. Comparison of the 2°-293/PERV regions
contained within the lambda clones with other PERV isolates

Region	Sequence[a]	Percent Identity			
		PERV-MSL[b]	PERV-A	PERV-1.15	GALV[c]
LTR	N	78			
5'-ut	N	97			
gag	N	98.9			
	A	98.5			62.6
pol	N	99			
	A	99			68
env	N	86.7	97	99.8	
	A	85	96	99.7	47
3'-ut	N	100			

[a] N, nucleotide sequence; A, amino acid sequence.
[b] PERV-MSL, a putative full-length PERV cDNA clone with the C-type envelope gene.
[c] GALV, gibbon ape leukemia virus.
ut untranslated region.

The *env* gene in lamA6 has 4 nucleotide and 2 amino acid differences with PERV-1.15, the 293/PERV *env* gene cloned by RT-PCR from virion RNA (Figure 18). Upon comparing the nucleotide sequence of the 293/PERV and PERV-MSL LTRs, a large divergence was observed in the U3 region (Figure 8). The 293/PERV LTR contains 71 more nucleotides than the PERV-MSL LTR. The sequence of the PERV sequences in Lambda clones A1, A11, A3A, A10, and A8 is also shown in Figure 18.

Two recombinant PERV proviruses were prepared to test if they produce infectious virus in 293 cells. The constructs, an A3A/A6 chimera and an A1/A6 chimera, are outlined in Figure 9. All constructs were built in the pBluescript KS vector (Stratagene), and used the unique BspEI site in the *pol* gene to link the 3'-half of the genome contained in lam A6 to the 5'-half of the genome contained in either lamA1 or lamA3A. A minimum of genomic sequence was cloned with the PERV region: A1, about 1000 bp; A3A, about 30 bp; A6, about 700 bp.

Example 4

Preparation and Detection of Anti-PERV Antibodies

Rabbit polyclonal antisera is produced against 2°-293/PERV virion proteins and against 2°-293/PERV capsid proteins. The 2°-293/PERV cultures

5   produce a relatively large amount of viral particles as demonstrated in Figure 10A. Goat anti-SSAV CA and goat anti-GALV CA antisera easily detect PERV Gag proteins on a Western blot with only minor cross-reaction to control 293 cell supernatant proteins (Figure 10B). These antisera are used as positive controls for the characterization of the anti-PERV antisera and monoclonal

10   antibodies. The 2°-293/PERV cultures produce approximately equal amounts of the p30 capsid (CA) protein in 5 ml of supernatant as ALV infected cultures produce ALV p27 CA protein (lanes 2 and 5). The high-titer ALV stock contained 10^7 iu/ml. The 2°-293/PERV virions are collected and purified by equilibrium density centrifugation on sucrose gradients. The banded virions are

15   collected, dialyzed, and used for antibody production. The PERV CA protein is isolated from SDS-PAGE gels (Figure 10A) and also used for polyclonal antisera production.

Two rabbits are used for each antigen preparation. The antigen (100 µg) mixed in Complete Freund's Adjuvant is used for the initial inoculation. The

20   animal is boosted with 50 µg antigen in Incomplete Freund's Adjuvant on day 14, 21 and 49 after the initial inoculation. The route of injection is normally subcutaneous and/or intramuscular at multiple sites. Test bleeds are drawn on day 35 and 56 and screened against PERV proteins by Western blot and immunoprecipitation assays.

25   Purified PERV CA protein is used to produce mouse hybridomas that secrete monoclonal antibodies against PERV CA. The PERV capsid protein, purified from polyacrylamide gels, is used as the antigen. Five Balb/c mice are injected intraperitoneally or subcutaneously with 400 µg antigen (per mouse) in Complete Freund's Adjuvant and boosted two weeks later with 250-500 µg of

30   the antigen in Incomplete Freund's Adjuvant. All five mice are then tested for the presence of PERV anti-capsid antibodies by Western blot. One of the positive mice is used for hybridoma preparation. Spleenocytes and/or lymph node cells are removed and fused to non-immunoglobulin secreting myeloma

cell line F/O. Hybridomas are screened and positive hybrids are cloned and then subcloned to obtain monoclonal antibody (MoAb) producing cell lines. The hybridomas are screened by both Western blot and immunoprecipitation of PERV capsid protein. The MoAb cell lines are isotyped and cryopreserved.

5   MoAbs are purified from the cell line supernatant by protein A/G column chromatography.

The new anti-PERV antibodies, as well as the anti-SSAV and anti-GALV CA antisera, are tested for their ability to detect PERV proteins in live and fixed PERV infected cells. An immunofluorescence and/or immunohistochemical

10   assay for PERV infection simplifies the detection of PERV infection and allows the direct quantitation of PERV titers.

To detect anti-PERV antibodies in sera, a Western-based assay system is employed. Protein preparations made from PERV virions produced by the 2°-293/PERV culture are separated by SDS-PAGE and transferred to nitrocellulose

15   filters. Virion protein extracts are used for two reasons. First, PERV produced by human cells does not display the α-Gal xenoreactive carbohydrate that may bind to the α-Gal antibodies in human sera. Second, the 2°-293/PERV culture produces high levels of PERV virions as shown by analysis of PERV virion protein extracts. Goat anti-SSAV and anti-GALV CA antisera that cross-reacts

20   with the PERV CA protein is employed. The 2°-293/PERV virions are purified by equilibrium centrifugation on sucrose gradients. In one embodiment, the assay employs peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Amersham, Pharmacia Biotech, Piscataway, NJ) to detect antibodies bound to the PERV proteins. To determine what effect human sera

25   may have on the performance of the assay (e.g., alter the antibody sensitivity or cross-reaction with common human antibodies), the anti-CA antisera is added to dilutions of control human sera (1:10, 1:50, 1:100) and tested.

Serum samples from at least 300 pig slaughterhouse workers, plus controls, are screened. Control samples, including sera from a patient with little

30   or no exposure to pigs, and sera from two populations of retrovirus-infected patient populations (50 HIV-1; 20 HTLV-1) is used to evaluate the specificity of antibody binding to PERV and non-PERV proteins. Preparative single well SDS-PAGE is used to separate the PERV virion proteins, and the proteins

transferred to a nitrocellulose filter. The Miniblotter II apparatus (Immunetics,

Cambridge, MA) permits the screening of 45 different samples by dividing the

Western blot into 45 sample cells. A negative control serum and a positive

control serum (human sera with SSAV or GALV anti-capsid antisera) is assayed

5   with each blot. A 1:50 dilution of the serum samples is screened initially.

Human blood samples are also tested for PERV nucleic acid and anti-PERV

antibodies.

Example 5

10   Soluble Forms of the Subgroup A Avian Leukosis Virus Receptor Tva

Significantly Inhibit ALV(A) Infection *in vitro* and *in vivo*

Three cell surface proteins have been identified as ALV receptors: Tva,

the receptor for ALV(A) (Bates et al., 1998; Bates et al., 1993; Young et al.,

1993); CAR1, the receptor for ALV(B) and ALV(D) (Brojatsch et al., 1996;

15   Smith et al., 1998); and SEAR, the receptor for ALV(E) (Adkins et al., 1997).

To aid in the characterization of the interactions between Tva and ALV(A)

envelope glycoproteins, soluble forms of the 83 amino acid extracellular domain

of the Tva receptor protein (sTva) were constructed by Young and colleagues

(Connolly et al., 1995), who reported that pre-incubation of the sTva proteins

20   with different envelope subgroup ALVs caused a specific block to infection of

susceptible chicken cells by ALV(A), but had no effect on ALV(B) or ALV(C)

infection.

To determine if cells and chickens expressing sTva proteins are resistant

to ALV(A) infection, ALV-based replication-competent retroviral vectors were

25   used to efficiently deliver and express *stva* genes (Federspiel et al., 1997). The

vectors are available with five different envelope subgroups (A-E) which enables

multiple genes to be delivered and expressed in virtually every cell.

Materials and Methods

Soluble receptor and retroviral vector constructs. The two soluble

30   receptor gene constructs, contained in the plasmids pLC126 and pKZ457, were

gifts of John Young (Harvard Medical School). The pLC126 *stva* gene

(Connolly et al., 1994; Hughes et al., 1987), encoding the 83-amino-acid Tva

extracellular domain fused to a 9-amino-acid antibody epitope tag derived from

influenza virus hemagglutinin, followed by six histidine residues, was isolated as

a *NcoI-PstI* fragment and cloned into the *NcoI* and *PstI* sites of the CLA12NCO

adaptor plasmid (Federspiel et al., 1997; Hughes et al., 1987). The pKZ457 *stva-mIgG* gene, encoding the 83-amino-acid Tva extracellular domain fused to the

5   constant region of the mouse IgG heavy chain (nucleotides 353-1072) (Tucker et

al., 1979), was isolated as a *NcoI-BlpI* fragment. The *BlpI* site was made blunt

and the modified fragment was cloned into the *NcoI* and *SmaI* sites of

CLA12NCO. Both the *stva* and *stva-mIgG* genes had been modified to contain

NcoI sites at their initiator ATGs. The soluble receptor gene cassettes were

10   isolated as *ClaI* fragments from the adaptor plasmids and cloned into the unique

ClaI site of the RCASBP, RCAS, and RCOSBP retroviral vectors with subgroup

(B) and subgroup (C) envelope genes. The RCAS family of replication-

competent retroviral vectors have been described (Federspiel et al., 1994;

Federspiel et al., 1997; Hughes et al., 1987; Petropoulous et al., 1991;

15   Petropoulous et al., 1992).

        The *stva-mIgG* gene isolated as a *ClaI* fragment from the CLA12NCO

adaptor plasmid was subcloned into the TFANEO expression vector (Federspiel

et al., 1989). TFANEO is a companion expression vector to the RCAS family of

retroviral vectors. The expression cassette of TFANEO consists of two LTRs

20   derived from the RCAS vector that provide strong promoter, enhancer, and

polyadenylation sites flanking a unique *ClaI* insertion site. The TFANEO

plasmid also contains a *neo* resistance gene expressed under the control of the

chicken β-actin promoter, and an ampicillin resistance gene for selection in *E.

coli.*

25   The RCASBP(A)AP, RCASBP(C)AP and RCASBP(C)AP retroviral

vectors which contain the heat-stable human placental alkaline phosphatase gene

(AP) have been described (Federspiel et al., 1997; Field-Berry et al., 1992;

Fekete et al., 1993). The AP gene, contained on a *SalI* fragment, was cloned into

the *SalI* site of the CLA12 adaptor plasmid, and then subcloned into the

30   RCASBP vectors as a *ClaI* fragment (gift of Constance Cepko).

        Cell culture and virus propagation. Chicken embryo fibroblasts (CEFs)

derived from 10-day, line 0 embryos (C/E) (Astrin et al., 1979) were grown in

DMEM (GIBCO/BRL) supplemented with 10% tryptose phosphate broth

54

(GIBCO/BRL), 5% fetal bovine serum (GIBCO/BRL), 5% newborn calf serum

(GIBCO/BRL), 100 units of penicillin per ml, and 100 µg of streptomycin per

ml (Quality Biological, Inc., Gaithersburg, MD) as previously described

(Federspiel et al., 1997). DF-1 cells were grown in DMEM supplemented with

5   10% fetal bovine serum, 100 units of penicillin per ml, and 100 µg of

streptomycin per ml (Himly et al., 1998; Schaefer-Klein et al., 1998). Both CEF

and DF-1 cultures were passages 1:3 when confluent.

Virus propagation was initiated by calcium phosphate transfection of

plasmid DNA that contained the retroviral vector in proviral form (Federspiel et

10   al., 1997). In standard transfections, 5 µg of purified plasmid DNA was

introduced into DF-1 cells or early passage CEF by the calcium phosphate

precipitation method (Kingston et al., 1989). Viral spread was monitored by

assaying culture supernatants for ALV capsid protein by either Western transfer

analysis or ELISA (Smith et al., 1979). Virus stocks were generated from the

15   cell supernatants. The supernatants were cleared of cellular debris by

centrifugation at 2000 × g for 10 minutes at 4°C and stored in aliquots at -80°C.

DF-1 cells transfected with the TFANEO plasmid were grown in 500 µg/ml

G418 (Gibco/BRL) to select for neomycin-resistant cells. Clones were isolated

using cloning cylinders (Bellco Glass Inc., Vineland, NJ), expanded, and

20   maintained with standard medium supplemented with 250 µg/ml G418.

ALV alkaline phosphatase challenge assay. In a direct AP challenge

assay, CEF or DF-1 cell cultures (about 30% confluent) were incubated with 10-

fold serial dilutions of the RCASBP/AP virus stocks for 36-48 hours at 39°C. In

a pre-absorption AP challenge assay, the 10-fold viral serial dilutions were first

25   mixed with 2 ml of supernatant containing sTva-mIgG for 3 hours at 4°C, and

then assayed as above. The assay for alkaline phosphatase activity was modified

from procedures of Cepko and co-workers (Federspiel et al., 1994; Fekete et al.,

1993; Fields-Berry et al., 1995). Cells were fixed in 4% paraformaldehyde in

Dulbecco's phosphate-buffered saline (PBS) for 30 minutes at 25°C, washed

30   twice in PBS for 5 minutes each, and incubated for 1 hour at 65°C to inactivate

endogenous AP activity. The cells were then washed twice with AP detection

buffer (100 mM Tris•Cl, pH 9.5/100 mM NaCl/50 mM MgCl$_2$) for 10 minutes

and exposed to the AP chromogenic substrates nitroblue tetrazolium (300 µg/ml)

and 5-bromo-4-chloro-3-indoyl phosphate (170 µg/ml) (GIBCO/BRL).

Enzymatically active AP produces an insoluble purple precipitate. The reaction was stopped by the addition of 20 mM EDTA, pH 8.0 in PBS.

Immunoprecipitation and Western transfer analysis of stva-mIgG

5   proteins. A 500 µl aliquot of culture supernatant or serum was incubated with 50 µl of anti-mouse IgG-agarose beads (Sigma) for ≥ 1 hour at 4°C. The sTva-mIgG agarose bead complexes were collected by centrifugation and washed twice in dilution buffer [50 mM Tris-buffered saline (TBS), 1% Triton X-100, 1 mg/ml BSA], once in 50 mM TBS and once in 0.05 M Tris•Cl, pH 6.8. The

10   washed complexes were collected by centrifugation, resuspended in 50 µl 1X Laemmli buffer (2% SDS, 10% glycerol, 0.05 M Tris•Cl, pH 6.8, 0.1% bromophenol blue) without β-mercaptoethanol, and heated for 5 minutes at 100°C. The agarose in the samples was collected by centrifugation for 2 minutes and the supernatants were transferred to new tubes. Prior to gel electrophoresis,

15   1.0 µl β-mercaptoethanol was added to each 50 µl sample and the samples were heated for 5 minutes at 100°C. The denatured immunoprecipitates were separated by 12% SDS-PAGE, and transferred to a nitrocellulose membrane. The filters were blocked with 10% non-fat dry milk (NFDM) in PBS, probed with 0.05 µg/ml peroxidase-conjugated goat anti-mouse IgG antibodies

20   (Kirkegaard and Perry laboratories, Gaithersburg, MD) in rinse buffer (100 mM NaCl, 10 mM Tris•Cl, pH 8, 1 mM EDTA, 0.1% Tween 20) and 1% NFDM, and washed in rinse buffer. Protein/antibody complexes were detected with the Western Blot Chemiluminescence Reagent (NEN) according to the manufacturer's instructions. The immunoblot was then exposed to Kodak X-

25   Omat film.

In vivo ALV challenge assay. Line 0 embryos were somatically infected with RCASBP(B), RCASBP(B)stva, or RCASBP(B)stva-mIgG by injecting unincubated eggs near the blastoderm with 100 µl containing 1×10^6 CEF or DF-1 cells producing the virus. Line 0 is a White Leghorn line that is

30   genetically susceptible to all ALV subgroups except subgroup (E) and is free of endogenous proviruses that are closely related with ALV (Astrin et al., 1979). Viremic chicks were identified at hatch by ELISA for the ALV capsid protein p27. Viremic and uninfected control chicks were infected intra-abdominally

with 10^5 infectious units of either RAV-1 [an ALV(A) isolate] or RAV-49 [an ALV(C) isolate]. Blood was collected at 2, 4 or 9 weeks post-challenge, and the serum assayed for infectious subgroup (A), (B), or (C) ALV by the *in vitro* ALV Assay (see below).

5   *In vitro* ALV assay. The presence of infectious ALV in chickens was determined by assaying the serum samples on a panel of cell lines with different ALV envelope subgroup susceptibilities. The panel of indicator cell lines included: line 0 CEF (C/E) which supports the replication of ALV(A), ALV(B), and ALV(C); line *alv6* CEF (C/A) which supports ALV(B) and ALV(C)

10   replication; line RP30B-cell line (C/B) which supports ALV(A) and ALV(C) replication; and line 15.C-12 CEF (C/C) which supports ALV(A) and ALV(B) replication. Serum samples (100 μl) were added to the cells and the cells were incubated for 9 days in media (5% serum) to allow ALV to spread. The media was changed after 3 days to avoid detection of ALV proteins in the original

15   serum sample. The cells were then solubilized by 2 cycles of rapid freeze-thaw to release ALV Gag antigens. The ALV capsid protein was detected by ELISA. A positive sample was defined as having an optical density reading of > 0.200. The *in vitro* ALV Assay can detect infectious ALV titers as low as 10 IFU/ml.

       RNase protection assay. Total RNA was isolated from cells in culture, or

20   homogenized tissues of experimental birds, by the RNazol B method (Tel-Test, Inc., Friendswood, TX). Sequence-specific RNA probes were cloned into pBluescript KS as follows: the RAV-1 envelope sequences were cloned as an *Xho*I to *Xba*I fragment (Genbank accession # M19113; nucleotides 248-676) (Bora et al., 1988); the RAV-2 envelope sequences were cloned as a *Bam*HI to

25   *Sal*I fragment (Genbank accession # M14902; nucleotides 612-1080) (Bora et al., 1986); the *stva* probe was generated from an *Eco*RI to *Pst*I fragment from the plasmid pLC126 (Genbank accession # L22752; nucleotides 9-386) (Bates et al., 1993; Connolly et al., 1994); and the *stva-mIgG* probe was generated from a *Cla*I to *Bam*HI fragment derived from the adaptor plasmid construct which

30   contains the 5'-*Cla*I site and transcription leader from the CLA12NCO adaptor plasmid and a synthetic sequence encoding the 83-amino acid Tva extracellular domain from pKZ457 that is different from the *stva* gene. A fragment of the chicken glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (Genbank

accession # K01458; nucleotides 163-361) (Panabieres et al., 1984) was used as

a control for the quantity and quality of the RNA. The constructs were

linearized by restriction endonuclease digestion and gel purified. ^{32}P-labeled

antisense RNA probes were synthesized using the RNA Transcription Kit

5   (Stratagene, La Jolla, CA). The probes were hybridized with 20 µg of total RNA

in 20 µl hybridization solution (80% formamide, 10 mM sodium citrate pH 6.4,

300 mM sodium acetate pH 6.4, 1 mM EDTA) overnight at 42°C. RNase

protection assays were performed using the RPA II Ribonuclease Protection Kit

(Ambion, Austin, TX). The RNA samples were digested with the RNase A/T1

10   mixture diluted 1:75. The protected RNA probe fragments were separated on a

6% acrylamide/7.6 M urea gel and exposed to Kodak X-Omat film.

PCR assays. DNA was isolated from cells in culture or tissues of

experimental birds using the QIAmp Tissue Kit (Qiagen). Each PCR contained

1.25 µl 10X PCR buffer (final concentration, 50 mM Tris•Cl, pH 8.3, 50 mM

15   KCl, 7 mM MgCl$_2$, 1.1 mM β-mercaptoethanol), 1.25 µl of 1.7 mg/ml BSA, 0.5

µl of each dNTP at 25 mM, 0.5 µl of each primer (A$_{260}$ = 5), 6.0 µl H$_2$O, and 1.0

µl of DNA (genomic DNA about 100 ng/µl; plasmid DNA about 2 ng/µl). The

reactions were heated to 90°C for 1 minute and initiated by the addition of 1.5 µl

of Taq DNA polymerase (Promega, Madison, WI) diluted 1:10 v/v (0.75 units).

20   Thirty cycles of PCR were carried out as follows: 90°C for 40 seconds, then

59°C for 80 seconds. Diagnostic primers used to detect ALV(A) *env* (Bora et

al., 1988) were 5'-GGGACGAGGTTATGCCGCTG-3' (SEQ ID NO:24; about

50 bp upstream of *Kpn*I site) and 5'-GGGCGTGCGCGCATTACCAC-3' (SEQ

ID NO:25; nucleotides 871-851), yielding a 937 bp fragment. The PCR

25   extension temperature was increased to 62°C for amplifying ALV(A) *env*.

Diagnostic primers used to detect ALV(B) *env* (Bora et al., 1986) were 5'-

GACCGACCCAGGGAACAATC-3' (SEQ ID NO:26; nucleotides 713-732)

and 5-ATGAGGAAAATTGCGGGTGG-3' (SEQ ID NO:27; nucleotides 1141-

1122), yielding a 429 bp fragment. Diagnostic primers used to detect *stva* (Bates

30   et al., 1993; Connolly et al., 1994) were 5'-GGAATGTGACTGGTAATGGA-3'

(SEQ ID NO:28; nucleotides 56-75) and 5'-GCCTTAGTGATGGTGATGGT-3'

(SEQ ID NO:29; nucleotides 369-350), yielding a 314 bp fragment. Diagnostic

primers used to detect *stva-mIgG* were 5'-CCATCCGTCTTCATCTTCCCT-3'

(SEQ ID NO:30; nucleotides 974-994) and 5'-

TGGTGCGGTGTCCTTGTAGTT-3' (SEQ ID NO:31; nucleotides 1562-1542),

yielding a 589 bp fragment of the mouse IgG gene (Tucker et al., 1979). The

amplified DNA fragments were separated on 0.8% agarose gels and visualized

5   with ethidium bromide.

Results

Experimental approach. The *stva* and *stva-mIgG* receptor gene fusions

were subcloned into the CLA12NCO adaptor plasmid which contains a

transcriptional leader sequence and has a consensus ATG start site contained in a

10   *Nco*I site. These sequences work very efficiently with the promoter/enhancer

elements of the ALV-based retroviral vectors to express experimental genes at

high levels (Hughes et al., 1987). The RCAS family of retroviral vectors were

derived from the Schmidt-Ruppin A strain of Rous sarcoma virus (RSV) and

were present in proviral form on pBR-based plasmids (Federspiel et al., 1997).

15   Experimental genes were inserted into the vectors in the unique *Cla*I site (which

replaces the *src* gene in RSV) and so are translated from a spliced mRNA.

Retroviral vectors that carry and express the *stva* and *stva-mIgG* genes are shown

schematically in Figure 11. Virus propagation was initiated by transfection of

plasmid DNA containing the retroviral vector into avian cells (Figure 12). The

20   culture was then passaged until a maximum viral titer was achieved (6-10 cell

passages depending on the vector (Federspiel et al., 1997). Because vectors that

use different receptors are available, this system can be used to deliver multiple

genes to virtually all cells in the culture (Givol et al., 1994). Cell cultures that

express sTva or sTva-mIgG from a subgroup (B) or (C) vector were

25   subsequently challenged with ALV(A) to quantitate the antiviral effect of the

sTva proteins.

Antiviral effect of sTva *in vitro* and *in vivo*. The initial experiments

testing the effects of soluble receptors on viral replication were done with the

sTva protein. The *stva* gene was introduced into the RCASBP vector, which

30   produces the highest titer viral stocks and the highest level of expression of an

experimental. To quantitate the antiviral effect of sTva on ALV(A) infection,

CEF fully infected with the vector alone [either RCASBP(B) or RCASBP(C)],

vectors that express the *stva* gene [RCASBP(B)stva or RCASBP(C)stva] or

uninfected CEF were challenged with RCASBP(A)AP, the subgroup (A)

RCASBP vector containing the human placental alkaline phosphatase reporter

gene (AP). The results of a representative assay are shown in Table 8. CEF

cultures producing sTva, either from RCASBP(B) or RCASBP(C), were > 100-

5   fold more resistant to infection by RCASBP(A)AP than cultures infected with

the vector alone. CEF cultures infected by the RCASBP(B) or RCASBP(C)

vector alone were 3-5-fold less susceptible to RCASBP(A)AP. The antiviral

effect of sTva was specific for RCASBP(A)AP infection, since infection by

viruses with other envelope subgroups were not inhibited (Table 8).

10

Table 8.
Relative resistance of CEF expressing sTVA or sTVA-mIgG to ALV infection.

	Challenge Virus		
	RCASBP(A)AP[a]	RCASBP(B)AP	RCASBP(C)AP
Uninfected CEF	1.5×10^6	2.1×10^5	8.4×10^4
RCASBP(B)	4.3×10^5 (4)[b]	ND[c]	2.0×10^4 (4.2)
RCASBP(B)stva	2.9×10^3 (517)	ND	2.1×10^4 (4.0)
RCASBP(C)	6.0×10^5 (3)	8.3×10^4 (2.5)	ND
RCASBP(C)stva	4.0×10^3 (375)	1.2×10^5 (1.8)	ND
RCASBP(C)stva-mIgG	1.5×10^3 (1000)	8.0×10^4 (2.6)	ND

[a]AP-human placental alkaline phosphatase reporter gene.
[b]The resistance of experimental and control CEF to ALV infection relative to
uninfected CEF is given in parentheses.
[c]ND-not done

25

The CEF cultures infected with RCASBP(B) and RCASBP(B)stva were

used to inoculate unincubated line 0 eggs to produce chicks viremic with

RCASBP(B) or RCASBP(B)stva. Viremic chicks produced in this manner are

tolerant to most ALV antigens since the early embryo was infected. The chicks

30   were challenged with 10^5 infectious units of RAV-1, an aggressive ALV(A)

strain, to quantitate the antiviral effect of sTva. Blood samples were collected

from representative birds of each group at 2 weeks post-challenge, and from all

birds 9 weeks post-challenge. The sera were assayed for ALV(A) and ALV(B)

by the *in vitro* ALV Assay. The results of the challenges are summarized in

60

~~Table 9. Ninety-six percent of the birds infects with the RCASBP(B) vector~~
alone and then challenged with RAV-1 produced ALV(A) at both experimental
time points as expected. However, 95% of the birds infected with
RCASBP(B)stva did not produce detectable levels of ALV(A). These results

5   demonstrate that sTva has a strong antiviral effect on ALV(A) infection both *in
vitro* and *in vivo*. These results also demonstrate the utility of using vectors with
different subgroups *in vivo* since experimental birds could be infected with both
the RCASBP(B) vector and RAV-1. However, the level of sTva expression
could not be quantitated since neither the hemagglutinin nor the histidine epitope

10   tags included on the sTva protein allowed efficient immunoprecipitation of the
protein.

Table 9. Chickens expressing sTva are resistant to ALV(A) infection.

	Virus Subgroup Detected					
	2 Weeks[a]			9 Weeks		
	A&B	B	A	A&B	B	A
Uninfected	0/10	0/10	0/10	0/9	0/9	0/9
RCASBP(B)	9/9	9/9	0/9	6/6	6/6	0/6
RCASBP(B)+RAV-1	10/10	10/10	9/10	15/15	15/15	15/15
RCASBP(B)stva	10/10	10/10	1/10	11/12	11/12	0/12
RCASBP(B)stva+RAV-1	10/10	10/10	0/10	20/20	20/20	1/20

[a]All of the birds in each group were not necessarily assayed at both time points.

__Antiviral effect of sTva-mIgG *in vitro*__. Although the tagged version of

25   sTva could not be immunoprecipitated efficiently, an sTva immunoadhesin
sTva-mIgG can be immunoprecipitated and quantitated.   sTva-mIgG consists of
the 83-amino acid Tva extracellular domain fused to the constant region of the
mouse IgG heavy chain. The *stva-mIgG* gene was introduced into the
RCASBP(C) vector. To quantitate the antiviral effect of sTva-mIgG compared

30   to sTva, CEF cultures infected with RCASBP(C)stva-mIgG, RCASBP(C)stva,
or RCASBP(C) were challenged with either RCASBP(A)AP or RCASBP(B)AP.
CEF expressing sTva-mIgG were about 300-fold more resistant to

~~RCASBP(A)AP infection compared to cells infected with the vector alone, and~~

2- to 3-fold more resistant than cells expressing sTva (Table 8). The antiviral effect was specific for ALV(A) since no significant change in susceptibility was observed when the cultures were challenged with RCASBP(B)AP.

5   ALV replication in a permanent, non-transformed cell line derived from line 0 CEF called DF-1 has been described (Himly et al., 1998; Schaefer-Klein et al., 1998). ALV and ALV-based retroviral vectors replicate and express inserted genes in DF-1 cells at levels similar to CEF, and DF-1 can be used to generate clonal cell lines. The antiviral effect of sTva-mIgG produced in DF-1 cultures

10   infected with RCASBP(C)stva-mIgG (Table 10) was similar to that seen in CEF cultures (Table 8). The sTva-mIgG protein was immunoprecipitated from cell culture supernatants with anti-mouse IgG antibody conjugated to agarose beads and analyzed by Western transfer of SDS-PAGE gels (Figure 13). The immunoprecipitated sTva-mIgG protein migrates as a broad band (50-60 kDa)

15   due to post-translational modification and as a minor about 38 kDa band. The about 38 kDa band is probably a degradation product of sTva-mIgG since both bands appear after immunoprecipitation with an ALV(A) surface glycoprotein immunoadhesin, and the amount of the about 38 kDa band increases after repeated freeze-thaw cycles of the viral supernatants. Stable clonal DF-1 cell

20   lines were generated that express different levels of sTva-mIgG under the control of the TFANEO expression vector. These cell lines do not produce infectious ALV and are resistant to RCASBP(A)AP infection at levels similar to cultures expressing sTva-mIgG from the retroviral vectors. Therefore, chronic ALV infection does not make a major contribution to the antiviral effect obtained.

25   Relationship between sTva-mIgG expression level and the antiviral effect. The *stva-mIgG* gene was subcloned into the RCAS(C) and RCOSBP(C) retroviral vectors. Previously, it has been reported that the RCAS vector replicates to a 5-15-fold lower titer (depending on envelope subgroup and inserted gene) compared to RCASBP and produces protein at an equivalently

30   reduced level (Federspiel et al., 1994). The RCOSBP vector, which lacks a strong transcription enhancer in the LTR, replicates to about 100-fold lower titer compared to RCASBP and produces lower levels of protein. DF-1 cultures infected with RCASBP(C)stva-mIgG, RCAS(C)stva-mIgG, or RCOSBP(C)stva-

~~mIgG were challenged with RCSABP(A)AP to determine the antiviral effect of~~
different levels of sTva-mIgG on ALV(A) infection. The results of a
representative assay are shown in Table 10. The sTva-mIgG produced by these
DF-1 cultures was detected by immunoprecipitation of sTva-mIgG followed by
5 the analysis of the proteins by Western transfer (Figure 13). As expected,
cultures infected with RCASBP produced the highest level of sTva-mIgG and
the greatest antiviral effect (about 200-fold). Cultures infected with RCAS
produced slightly lower levels of sTva-mIgG protein and a lower antiviral effect
(about 100-fold). Finally, cultures infected with RCOSBP produced the lowest
10 level of sTva-mIgG protein and a modest antiviral effect (about 15-fold).

Table 10.

Relative resistance of DF-1 cells expressing sTva-mIgG to ALV(A) infection.

	RCASBP(A)AP	Resistance[a]
Uninfected DF-1	3.3×10^6	---
RCASBP(C)	1.3×10^6	3
RCASBP(C)stva-mIgG	5.3×10^3	622
RCAS(C)	6.4×10^5	5
RCAS(C)stva-mIgG	5.7×10^3	579
RCOSBP(C)	9.0×10^5	4
RCOSBP(C)stva-mIgG	5.2×10^4	63

[a]The resistance of the cells to ALV(A) infection was determined by dividing the
titer   obtained on the control uninfected DF-1 cells by the titer obtained for each
     experimental group.

25   The antiviral effect of sTva and sTva-mIgG on ALV(A) infection may
represent the minimum antiviral effect attainable *in vitro* as measured by the
direct ALV AP challenge assay. The assays were done on subconfluent cell
cultures (30%) where the levels of the soluble receptor protein had not
accumulated to the levels expressed by a confluent culture. To determine the
30 antiviral effect of higher levels of sTva-mIgG, RCASBP(A)AP was pretreated
with supernatants collected from confluent DF-1 cultures infected with
RCASBP(B), RCASBP(B)stva-mIgG, RCOSBP(B), or RCOSBP(B)stva-mIgG,
and then assayed as before. Preabsorption of RCASBP(A)AP with high levels of

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~~sTva-mIgG significantly increased the antiviral effect compared to a direct~~
assay: RCASBP(B)stva-mIgG pretreatment increased the antiviral effect of the
direct assay about 500-fold; and RCOSBP(B)stva-mIgG pretreatment increased
the direct antiviral effect about 60-fold.

5   Delivery and expression of sTva and sTva-mIgG *in vivo*. To characterize
the efficiency of RCASBP delivery and expression of the sTva proteins in
chickens, unincubated line 0 eggs were injected with CEF producing the
RCASBP(B) or RCASBP(C) vectors alone, the vectors with the *stva* gene, or the
vectors with the *stva-mIgG* gene. Viremic chicks were identified on the day of
10   hatch by an ELISA assay for ALV capsid protein. The sTva-mIgG protein was
immunoprecipitated from serum samples of both RCASBP(B)stva-mIgG and
RCASBP(C)stva-mIgG infected birds and visualized by Western analysis
(Figure 14). The *stva*, *stva-mIgG*, and RCASBP(B) *env* RNA expression levels
in liver, heart, spleen, bursa, thymus, kidney, and muscle tissues of infected birds
15   were analyzed by RNase protection assay. An RNase protection analysis of a
representative bird infected with RCASBP(B)stva and a representative bird
infected with RCASBP(B)stva-mIgG are shown in Figure 15. Relatively high
levels of the *stva* or *stva-mIgG* and ALV(B) *env* RNAs were detected in all
tissues assayed, indicating that the inserted genes were delivered and expressed
20   efficiently by the RCASBP(B) vector.

Table 11.
Chickens expressing either sTva or sTva-mIgG are resistant to ALV(A) infection
but not ALV(C) infection.

	Virus Subgroup Detected[a]		
	B	A	C
RCASBP(B)+RAV-1	4/4	4/4	
RCASBP(B)stva+RAV-1	5/5	0/5	
RCASBP(B)stva-mIgG+RAV-1	9/9	0/9	
RCASBP(B)+RAV-49	4/4		3/4
RCASBP(B)stva+RAV-49	4/4		4/4
RCASBP(B)stva-mIgG+RAV-49	7/8		6/8

[a]Assays were done 4 weeks after challenge.

Antiviral effect of sTva-mIgG *in vivo*. Chicks infected with the
RCASBP(B) vector alone, RCASBP(B)stva, or RCASBP(B)stva-mIgG were
split into two groups and challenged with 10^5 infectious units of either RAV-1
(subgroup A) or RAV-49 (subgroup C). Blood was collected from each bird
four weeks after challenge, and the serum was assayed for ALV(A), ALV(B) and
ALV(C) by the *in vitro* ALV Assay (Table 11). As expected, ALV(B) was
detected in virtually all of the birds since the RCASBP(B) vector was used for
gene delivery. ALV(A) was not detected in the serum of RAV-1 challenged
birds containing the *stva* or the *stva-mIgG* genes. However, ALV(A) was
detected in the serum of the birds infected with the RCASBP(B) vector alone
and challenged with RAV-1. In contrast, the birds of all three experimental
groups were equally susceptible to RAV-49 challenge as shown by the presence
of ALV(C) in the majority of the birds. Since 19% of the birds challenged with
RAV-49 did not produce detectable levels of ALV(C), the titer of the RAV-49
stock may have been lower than expected. The antiviral effect of sTva and sTva-
mIgG was specific for ALV(A), consistent with the proposed mechanism of
antiviral action, receptor interference.

Representative birds from each RAV-1 challenged experimental group
were analyzed for the presence of ALV(A) and ALV(B) *env*, *stva* and *stva-mIgG*

~~sequences in RNA and DNA isolated from a variety of tissue samples of each~~
bird. A low level of RAV-1 infection and replication in a subset of tissues may
go undetected by the *in vitro* ALV assay due to virus inactivation by the sTva or
sTva-mIgG proteins in the serum. Antisense riboprobes and primer sets were

5   developed to specifically detect each target sequence by RNase protection assay
and PCR. RNA and DNA were isolated from liver, heart, spleen, bursa, thymus,
kidney, and muscle tissue of each bird and analyzed by RNase protection assay
and PCR assay. A representative RNase protection assay of RNA of one tissue
(bursa) from a bird of each experimental group, and an uninfected control bird, is

10   shown in Figure 16. A representative PCR analysis of DNA isolated form
tissues of a RAV-1 challenged bird from each experimental group is shown in
Figure 17. RAV-1 RNA and DNA were only detected in tissues of birds
infected with the RCASBP(B) vector challenged with RAV-1. Therefore, the
expression of sTva and sTva-mIgG significantly reduces, if not eliminates,

15   infection by the ALV(A) strain RAV-1 in chickens.

Discussion

Cells expressing the sTva proteins showed significant resistance to
ALV(A) infection, presumably due to the secreted receptor proteins binding the
glycoproteins of the invading virion, blocking the interactions of the virus and

20   the membrane bound Tva, a form of receptor interference. Tva has been
hypothesized to be necessary and sufficient to mediate ALV(A) entry (Balliet et
al., 1998; Bates et al., 1993). Several possible mechanisms could account for the
sTva inhibition of ALV(A) entry sTva binding of an ALV(A) surface
glycoprotein may lead to an irreversible conformational change in SU and TM.

25   Several studies have shown that sTva binding to purified ALV(A) envelope
glycoproteins induces a temperature-dependent conformational change in the
glycoproteins, and appears to convert the envelope glycoproteins to a membrane-
binding state (Balliet et al., 1999; Damico et al., 1999; Gilbert et al., 1995;
Hernandez et al., 1994). Binding of sTva or sTva-mIgG to the envelope

30   glycoproteins on the surface of the virus induces a conformational change in
both SU and TM similar to the events leading to fusion of the viral and host cell
membranes, and converts SU and TM into a form that is unable to bind Tva on
the surface of the cell. A conformational change may also lead to the loss of

some of the SU subunits (Orloff et al., 1993). Finally, sTva may inhibit ALV(A)
entry by simply binding to SU and physically blocking the access of membrane
bound Tva to the virion. By whatever mechanism(s), the sTva proteins block the
entry of ALV(A) into cultured cells and cells and tissues of chickens.

5   The replication-competent ALV-based retroviral vector experimental
system enabled the efficient delivery and expression of the *stva* and *stva-mIgG*
genes both in cultured cells and in virtually all the cells and tissues of the
chicken. RCASBP(A) and (RCASBP(B), as well as other combinations of ALV
retroviral vectors [ALV(B) followed by ALV(A); ALV(C) followed by

10   AVL(A)], can be used in CEF and DF-1 cells *in vitro* and *in vivo*. As reported
previously, the replication of some RCASBP(B) viruses in CEF and DF-1 cells,
and RCASBP(C) viruses on DF-1 cells, were somewhat cytopathic (Himly et al.,
1998; Schaefer-Klein et al., 1998). The cytopathic effect manifests itself as a
pause in growth rate (2-6 days), after which the cells recover, divide at a normal

15   rate, and express the viral and experimental proteins. It appears that only the
cells that produce high levels of the RCASBP(B) or RCASBP(C) envelope
glycoproteins show the cytopathic effects. The replication of subgroup (B)
and(C) RCAS and RCOSBP viruses, which replicate to lower titers compared to
RCASBP, do not cause detectable cytopathic effects. Chronic infection of CEF

20   and DF-1 cells with an ALV vector results in a low level of resistance to
infection by other ALV *env* subgroup vectors (2-5-fold) compared to uninfected
cells. This may indicate that while the ALV glycoproteins specifically and
efficiently interact with the appropriate receptor resulting in receptor
interference, the high level expression of one type of envelope glycoprotein on

25   the cell surface may interfere either directly or indirectly with the ability of other
ALVs to interact with their host receptors.

Both the RCASBP(B) and RCASBP(C) retroviral vectors were efficient
in generating viremic chicks without detectable pathologic effects in short term
infections, and the infected chicks expressed relatively high levels of sTva-mIgG

30   protein in their serum. Chicks infected with RCASBP(B) were also efficiently
infected with ALV(A). The RCASBP(B) vector efficiently delivered and
expressed the *stv-a* and *stva-mIgG* genes in all tissues tested, and resulted in a
significant antiviral effect on ALV(A) infection and replication. By delivering

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the *stva* genes in the early embryo, the immune system of the chicken can be

evaded. The birds are tolerized to the sTva and sTva-mIgG proteins, and to most of the ALV antigens since the viral vector used to deliver *stva* and *stva-mIgG* and the challenge viruses are virtually identical except for small regions of SU.

5   The expression of the sTva and sTva-mIgG proteins *in vivo* allowed the examination of the effects of the viral glycoprotein-soluble receptor interactions in a wide variety of cells. One RAV-1 challenged RCASBP(B)stva infected bird did contain infectious ALV(A) in its serum. Unfortunately the bird died of non-viral causes before tissues could be obtained.

10   CD4, an important cell-surface protein of the T-lymphocytes, is the primary receptor for HIV-1. Several groups developed and expressed soluble forms of CD4 (sCD4) and demonstrated that recombinant sCD4 proteins could bind specifically to HIV-1 envelope glycoproteins and inhibit HIV-1 infection *in vitro* (Daar et al., 1990; Harbison et al., 1990; Klasse et al., 1993; Orloff et al.,

15   1993; Schacker et al., 1995; Weiss, 1992). For injection of a recombinant antiviral protein to be effective against cell to cell transmission of the virus, it may be necessary to use a gene therapy approach in which target cells actively express the soluble receptor. The gene therapy approach has been tested for HIV-1: a sCD4 gene construct was expressed by a murine leukemia virus-based

20   retroviral vector in human T-cell lines and in primary peripheral blood lymphocytes (Morgan et al., 1994). In cell culture populations engineered to express sCD4 (30-50% of the cells contained the sCD4 gene) HIV-1 replication was inhibited 50-70% indicating that a sCD4 antiviral approach against HIV-1 might be more effective. Since the initial sCD4 studies were published, the

25   chemokine receptors have been identified as co-receptors necessary for efficient HIV-1 entry into cells (Hunter, 1997). Since both CD4 and a chemokine receptor are required for efficient HIV-1 entry into cells, sCD4 alone may not be an effective inhibitor of HIV-1 entry.

The results clearly indicate that a soluble receptor interference antiviral

30   strategy can effectively block the replication of at least some retroviruses, and that this approach may be applicable to other virus groups that require specific viral glycoprotein-host receptor interactions for entry into the cell. The application of this strategy to protect animals against specific viral diseases is

relatively straightforward since transgenic technology can be used to introduce
genes into animals and the transgenes will produce the desired protein without
provoking an immune response.

5   **Example 6**

Selection of Subgroup A Avian Leukosis Virus Mutants Resistant to the
Receptor

Interference Imposed by Soluble TVA and SUA

Genes encoding soluble forms of the subgroup A avian leukosis virus
10   (ALV) receptor Tva (sTva) and soluble envelope surface glycoprotein (SUA),
delivered by ALV-based retroviral vectors to cells, significantly inhibit ALV(A)
infection of those cells. The antiviral effects of sTva and SUA are consistent
with a receptor interference (RI) mechanism; the antiviral effect is specific for
ALV(A) since the susceptibility to infection by a neutral subgroup remains
15   unchanged. A virus-free stable cell line was developed which expressed either
sTva or SUA and so was significantly resistant to ALV(A) infection, 50-350-fold
and 1,000 to 200,000-fold, respectively. Since the SU region of Env determines
receptor usage, mutations capable of overcoming the RI may occur in the SU
region of the *env* gene of ALV(A) and would affect interactions between Tva
20   and Env(A).

ALV(A) was passaged on the sTva and SUA expressing cell lines.
Variant viruses capable of more rapid growth on the sTva cell lines were
identified at passage 7 and on the SUA cell lines at passage 9 post-infection.
The SU region of the *env* gene was PCR amplified from these cells, cloned and
25   analyzed by sequencing. Selection on sTva expressing cells produced mutant
viruses which were identical in sequence to ALV(A) except that 50% of the
clones screened had an amino acid change at codon 142, while the other 50%
had an amino acid change at codon 149 of *env*. All mutants selected on SUA
expressing cell lines contained a 6 amino acid deletion at codons 155-160 within
30   *env*. To determine if these mutations were sufficient to confer enhanced growth
on the resistant cell lines, the SU region of *env* containing the mutation, from
Kpn I to Sal I, was introduced into a wild-type ALV(A) molecular clone.

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~~Transfection of these mutants showed an enhanced rate of growth as compared~~
to wild-type.

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All publications, patents and patent applications are incorporated herein by reference. While in the foregoing specification this invention has been described in relation to certain preferred embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the detailed herein may be varied considerably without departing from the basic principles of the invention.

~~WHAT IS CLAIMED IS:~~

1. A method to inhibit or prevent infectious agent transmission in a
    mammalian transplant recipient, comprising:
    a) introducing to donor swine cells a recombinant DNA comprising
        a promoter operably linked to a DNA segment encoding a protein
        comprising at least a portion of a polypeptide of the infectious
        agent that is present in the extracellular form of the agent so as to
        yield transformed swine cells; and
    b) introducing the transformed swine cells to the recipient.

2. A method to inhibit or prevent infectious agent transmission in a
    mammalian transplant recipient, comprising:
    a) introducing to donor human blood cells a recombinant DNA
        comprising a promoter operably linked to a DNA segment
        encoding a protein comprising at least a portion of a polypeptide
        of the infectious agent that is present in the extracellular form of
        the agent so as to yield transformed human blood cells; and
    b) introducing the transformed human blood cells to the recipient.

3. A method to inhibit or prevent infectious agent transmission to a
    mammalian transplant recipient, comprising:
    a) introducing to a donor organ a recombinant DNA comprising a
        promoter operably linked to a DNA segment encoding a protein
        comprising at least a portion of a polypeptide of the infectious
        agent that is present in the extracellular form of the agent so as to
        yield a transformed organ; and
    b) introducing the transformed organ to the recipient.

4. The method of claim 1, 2 or 3 wherein the DNA segment encodes a
    fusion protein comprising at least a portion of a polypeptide of the
    infectious agent that is present in the extracellular form of the agent and
    a degradative enzyme.

5. ~~The method of claim 4 wherein the degradative enzyme is a nuclease or~~
protease.

6.   The method of claim 1, 2 or 3 wherein the infectious agent is a virus.

5

7.   The method of claim 6 wherein the polypeptide of the infectious agent is
a viral capsid protein, viral glycoprotein or an accessory protein.

8.   The method of claim 6 wherein the virus is a lentivirus, retrovirus,
10   hepatitis virus or a herpesvirus.

9.   The method of claim 1 wherein the donor cells are embryonic stem cells,
blood cells, neuronal cells, liver cells, pancreatic cells, kidney cells or
islet cells.

15

10.   The method of claim 3 wherein the organ is a heart, liver or kidney.

11.   The method of claim 3 wherein the organ is a human or pig organ.

20   12.   The method of claim 4 wherein the DNA segment encodes a fusion
protein encoding a polypeptide of a pig endogenous retrovirus.

13.   The method of claim 5 wherein the enzyme is barnase, staphylococcal
nuclease, RNase H1, RNase T1, retroviral protease, RNase III, RNaseL,
25   or a ribozyme.

14.   The method of claim 7 wherein the polypeptide of the infectious agent is
Vpr, Vpx, Vif or Nef.

30   15.   An isolated and purified nucleic acid molecule comprising a nucleic acid
segment which comprises at least a portion of a pig endogenous
retrovirus, wherein the nucleic acid segment hybridizes under hybridizing

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~~conditions to SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID~~
NO:21, SEQ ID NO:22, SEQ ID NO:23, or SEQ ID NO:32.

16.   An isolated and purified polypeptide encoded by a nucleic acid molecule comprising a nucleic acid segment comprising SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, or SEQ ID NO:32.

17.   A method to detect human tropic pig endogenous retroviruses, comprising:

      a)   contacting a mammalian sample suspected of being infected with a pig endogenous retrovirus with a probe comprising at least a portion of SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, or SEQ ID NO:32 so as to form complexes; and

      b)   detecting or determining the presence of the complexes.

18.   A method of using an isolated and purified nucleic acid molecule comprising the genome of a pig endogenous retrovirus comprising: introducing to a host cell a recombinant DNA molecule comprising a promoter operably linked to a DNA segment comprising SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, or SEQ ID NO:32 so as to yield a transformed host cell, and identifying the transformed host cell.

19.   The method of claim 1, 2, 3 or 18 wherein the recombinant DNA molecule further comprises transcriptional termination sequences 3' to the DNA segment.

20.   A host cell, the genome of which is augmented with a recombinant DNA molecule comprising a promoter operably linked to a DNA segment encoding a fusion protein comprising at least a portion of a polypeptide of a pig endogenous retrovirus and a degradative enzyme.

21.   ~~A fusion protein, comprising: a capsid or envelope protein of a porcine~~
        endogenous virus and a degradative enzyme.

22.   The fusion protein of claim 21 wherein the enzyme is a nuclease.

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23.   The fusion protein of claim 21 wherein the enzyme is a protease.

24.   The fusion protein of claim 21 wherein the enzyme is a lipase.

10   25.   The fusion protein of claim 21 wherein the activity of said enzyme is
        calcium-dependent.

26.   The fusion protein of claim 25 wherein the enzyme is staphylococcal
        nuclease.

15

27.   An isolated and purified DNA molecule encoding the fusion protein of
        claim 21.

28.   A recombinant virus comprising a nucleic acid molecule encoding the
20   fusion protein of claim 21.

29.   An antibody that specifically binds pig endogenous retrovirus.

30.   The antibody of claim 29 which binds a viral capsid protein.

25

31.   The antibody of claim 29 which binds the viral envelope glycoprotein.

32.   The method of claim 8 wherein the virus is Epstein Barr virus.

30   33.   The method of claim 8 wherein the virus is cytomegalovirus.

34.   The method of claim 8 wherein the virus is human immunodeficiency
        virus.

35. The method of claim 1, 2, 3 or 18 wherein the recombinant DNA is introduced to the recipient by infection with a recombinant virus.
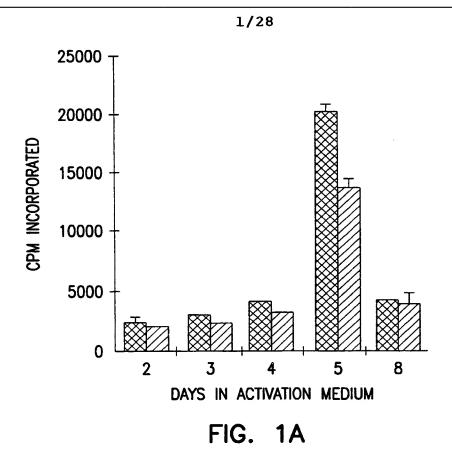
36. A method to inhibit or prevent infectious agent transmission in a mammalian transplant recipient, comprising:
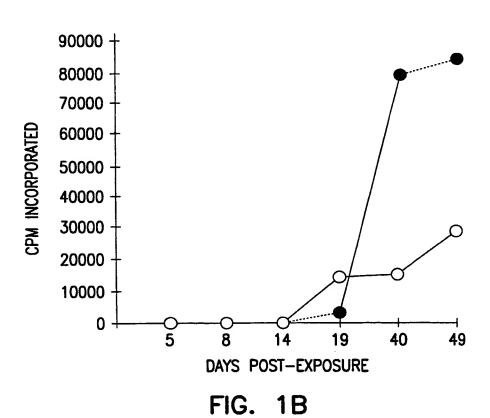
    a) introducing to donor mammalian cells a recombinant DNA comprising a promoter operably linked to a DNA segment encoding a protein comprising at least a portion of a polypeptide of the infectious agent that is present in the extracellular form of the agent so as to yield transformed mammalian cells; and

    b) introducing the transformed mammalian cells to the recipient.

37. A method to inhibit or prevent infectious agent transmission in a mammalian transplant recipient, comprising:

    a) introducing to donor human cells a recombinant DNA encoding a protein comprising at least a portion of a polypeptide of the infectious agent that is present in the extracellular form of the agent so as to yield transformed human cells; and

    b) introducing the transformed human cells to the recipient.

38. The method of claim 36 or 37 wherein the infectious agent is a virus.

39. The method of claim 38 wherein the polypeptide of the infectious agent is a viral capsid protein, viral glycoprotein or an accessory protein.

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FIG. 1A

FIG. 1B
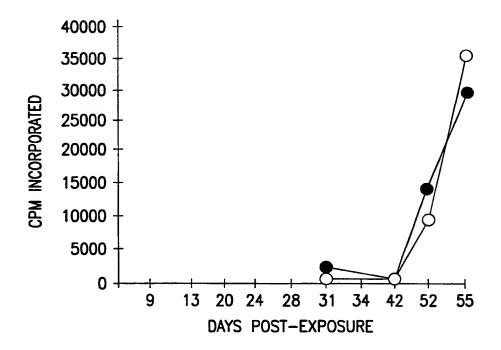
FIG. 1C
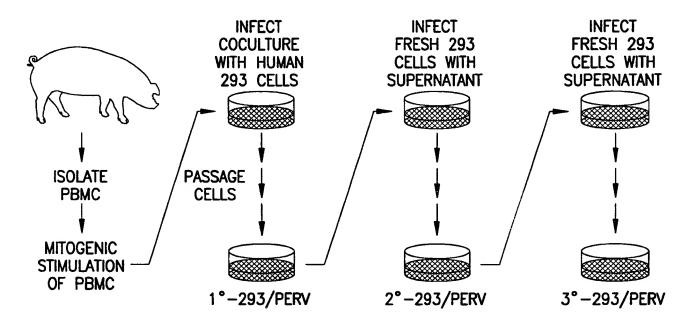
FIG. 2

FIG. 3

FIG. 4

FIG. 5

FIG. 6A

```
PERV-1.15  MHPTLSRRHL PIRGGKPKRL KIPLSFASIA WFLTLSITPQ VNGKRLVDSP   50
PERV-A     ......N... .......... .......... .........S T..M.IG..L   50
PERV-C     .......... .......... .......... .......... ..........   50

PERV-1.15  NSHKPLSLTW LLTDSGTGIN INSTQGEAPL GTWWPELYVC LRSVIPGLND  100
PERV-A     .......... .....I.... .......... ....D..... ..........  100
PERV-C     .......... .......... ....N..... .......... ......S.TS  100

PERV-1.15  QATPPDVLRA YGFYVCPGPP NNEEYCGNPQ DFFCKQWSCV TSNDGNWKWP  150
PERV-A     ....I.H... H......... ........I. I......... ..Y.......  150
PERV-C     ---...I.H. .GKH....R. .......... .......N.. ..........  147

PERV-1.15  VSQQDRVSYS PVNNPTSYNQ FNYGHGRWKD WQQRVQKDVR NKQISCHSLD  200
PERV-A     .T....F... Y.TY.SG... .......... ........I. ......Y...  200
PERV-C     T.....F... Y.TY.SG... ---------- ----LTWI-- TGSPK.SPS.  185

PERV-1.15  LDYLKISFTE KGKQENIQKW VNGMSWGIVY YRGSGRKKGS VLTIRLRIET  250
PERV-A     .......... ......L... .I..M..... .G.....KQP I.........  250
PERV-C     .......... .......... .......... .G.....KQP I....K.N-.  234

PERV-1.15  QMEPPVAIGP NKGLAEQGPP IQEQRPSPNP SDYNTTSGSV PTEPNITIKT  300
PERV-A     .L..M..... .......... .......... .......... ...S.S.T.M  300
PERV-C     .......... .TV.TG.R.. T.---G.G.. ..-S.I...D ..........  279

PERV-1.15  GAKLFNLIQG AFQALNSTTP EATSSCWLCL ASGPPYYEGM ARGGKFNVTK  350
PERV-A     S......... .......... .......... .......... ....R.....  350
PERV-C     .S........ .......... .......... .......... ....R.....  329

PERV-1.15  EHRDQCTWGS QNKLTLTEVS GKGTCIGMVP PSHQHLCNHT EAFNRTSESQ  400
PERV-A     .......... .......... .......... .......... ..........  400
PERV-C     .......... .......... .......K.. .......... .......Q..  379
```

FIG. 6B

```
PERV-1.15  YLVPGYDRWW  ACNTGLTPCV  STLVFNQTKD  FCVMVQIVPR  VYYYPEKAVL   450
PERV-A     ..........  ..........  ..........  .....I....  ........I.   450
PERV-C     ..........  ..........  ..........  ..........  ..........   429

PERV-1.15  DEYDYRYNRP  KREPISLTLA  VMLGLGVAAG  VGTGTAALIT  GPQQLEKGLS   500
PERV-A     ..........  ..........  ..........  .......V..  .....T....   500
PERV-C     ....NH.Q..  ..........  ..........  ..........  ..........   479

PERV-1.15  NLHRIVTENL  QALEKSVSNL  EESLTSLSEV  VLQNRRGLDL  LFLKEGGLCV   550
PERV-A     .......D..  ..........  ..........  ..........  ..........   550
PERV-C     .......D..  ..........  ..........  ..........  ..........   529

PERV-1.15  ALKEECCFYV  DHSGAIRDSM  NKLRERLEKR  RREKETTQGW  FEGWFNRSPW   600
PERV-A     ..........  ..........  S......R..  ...R.AD...  .......L..   600
PERV-C     ..........  ..........  ..........  ..........  ..........   579

PERV-1.15  LATLLSALTG  PLIVLLLLT   VGPCIINKLI  AFIRERISAV  QIMVLRQQYQ   650
PERV-A     MT........  ..V......   ...L..RFV.  ..V..V....  ..........   650
PERV-C     ..........  .........   ..........  ..........  ..........   629

PERV-1.15  SPSSREAGR    659
PERV-A     GLL.QGETDL   660
PERV-C     .........    638
```

FIG. 6B (CONTINUED)

FIG. 7

```
                →U3
        LamA1    AATGAAAGGA  TGAAAATGCA  ACCTGACTCT  CCCAGAACCC  AGGAAGTTAA   50
        PERV-MSL ..........  ........A..  ----------  ----------  ----...C...  31

        LamA1    TAAGAAGCTC  TAAATGCCCT  CGAATTCCAG  ACCCTGTTCC  CTATAGGTAA  100
        PERV-MSL .G.....T    A....TGTTC  T.........  .GTT......  T.........   81

        LamA1    AAGATCATAC  TTTTTGCTGT  TTTAAAATAT  GCTTTCTGCT  CTGTACAAAA  150
        PERV-MSL ....T.GGT   ..........  ..........  ----------  ----------  113

        LamA1    CTTTGTGGAA  GGGGAAAAAC  AGGCCCCTGA  GTATGTGCCT  CTATGCTTGA  200
        PERV-MSL -------G    AA.T....T   ..........  ...CA..T..  ...G.A...   153

        LamA1    AACTTCTTGA  AACTGCTCCT  AACTGCTTGT  TTGGCTTCTG  TAAACCTGCT  250
        PERV-MSL ..........  ....AT.TGA  G.TAA.AA.A  AAA.GGAG.T  .CT.A.....  203

        LamA1    TGCATAAGAT  AAAAAGAGGA  GAAGTCAATT  GCCTAACGGA  CCCCAGTAAG  300
        PERV-MSL .TT..GCT.   C-----T.T   A..ACTGG..  .GCC.TAA.   ----------  237

        LamA1    ATCGGGTGTA  CCACAAAATG  TTGAAACACA  TATCTTGGTG  ACAACATGTC  350
        PERV-MSL ----.A....- ---TG.....  T......     ..........  ..........  279

        LamA1    TCCCCCACCC  CGAAACATGC  GCAAATGTGT  AACTCTAAAA  CAATTTAAAT  400
        PERV-MSL ..........  ..........  ..........  ..........  ..........  329

        LamA1    TAATTGGTCC  ACGAAGCGCG  GGCTCTCGAA  GTTTTAAATT  GACTGGTTTG  450
        PERV-MSL ..........  ..........  ..........  ..........  ..........  379

        LamA1    TGATATTTTG  AAATGATTGG  TTTGTAAAGC  GCGGGCTTTG  TTGTGAACCC  500
        PERV-MSL ..........  ..........  ..........  ..........  ..........  429
```

FIG. 8

```
                                                                              U3←|→R
LamA1      CATAAAAGCT   GTCCCGACTC   CACACTCGGG   GCCGCAGTCC   TCTACCCCTG   550
PERV-MSL   .........    .........    .........    .........    .........    479

LamA1      CGTGGTGTAC   GACTGTGGGC   CCCAGCGCGC   TTGGAATAAA   AATCCTCTTG   600
PERV-MSL   .........    .........    .........    .........    .........    529

                        R←|→U5                                  U5←|→tRNA^Pro
LamA1      CTGTTTGCAT   CAAGACCGCT   TCTCGTGAGT   GATTAAGGGG   AGTCGCCTTT   650
PERV-MSL   .........    .........    .........    .........    .........    579

LamA1      TCCGAGCCTG   GAGGTTCTTT   TTGCTAGTCT   TACATTTGGG   GGCTCGTCCG   700
PERV-MSL   .........    .........    .G......     .........    .........    629

           ←|
LamA1      GGAT                                                             704
PERV-MSL   ....                                                             633
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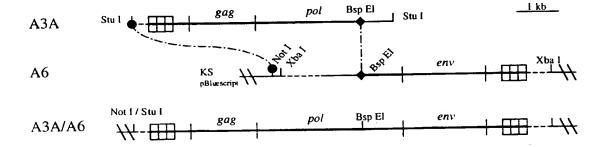
FIG. 8 (CONTINUED)

FIG. 9A
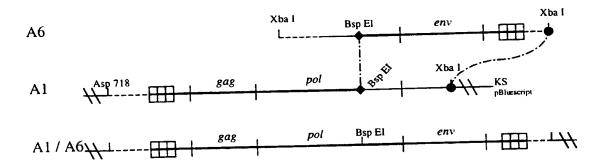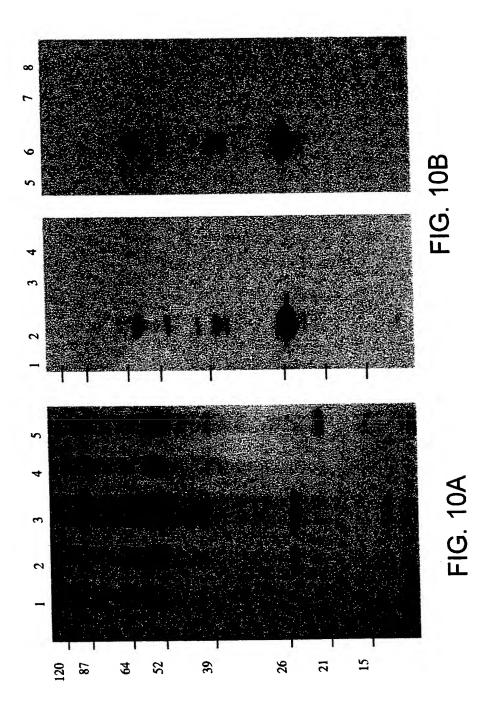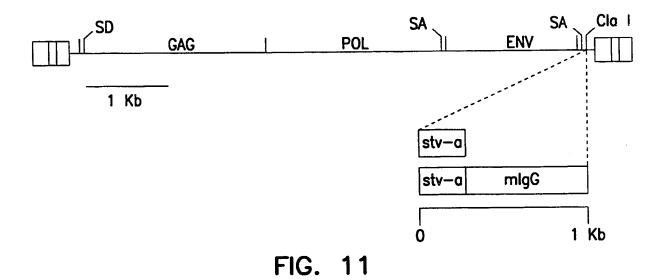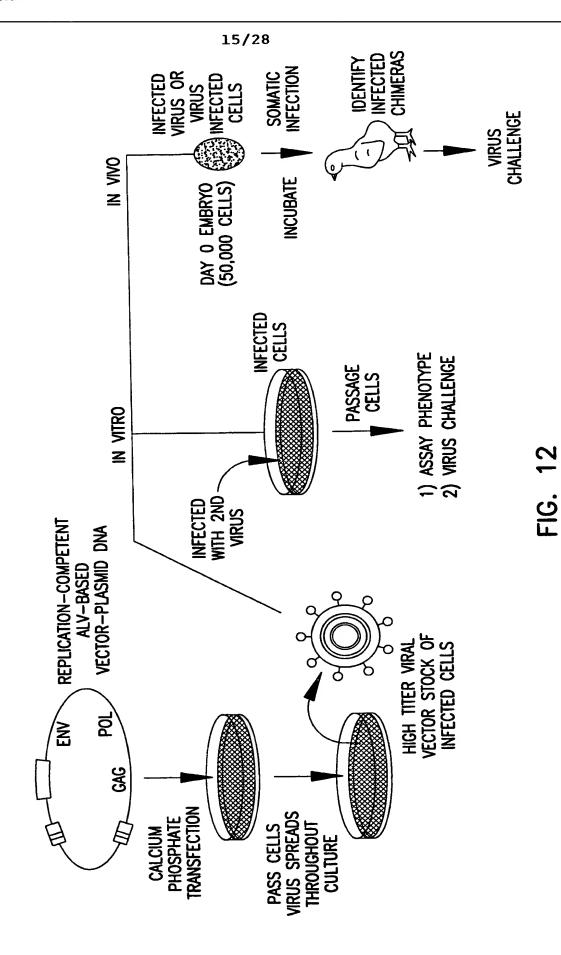
FIG. 9B

FIG. 10B

FIG. 10A

FIG. 11

FIG. 12

FIG. 13

FIG. 14

FIG. 15B

RCASBP(B)stva-mIgG

FIG. 15A

RCASBP(B)stva

FIG. 16D

FIG. 16C

FIG. 16B

FIG. 16A

FIG. 17
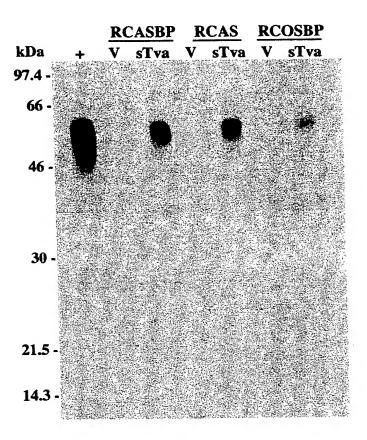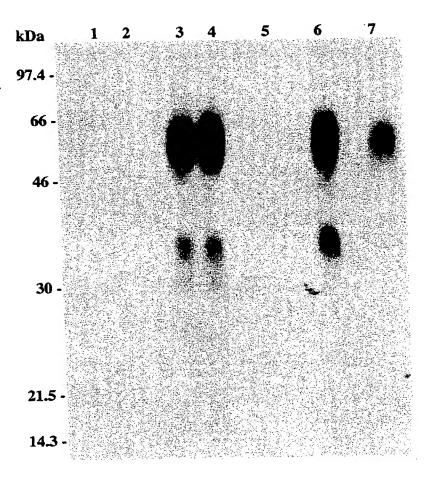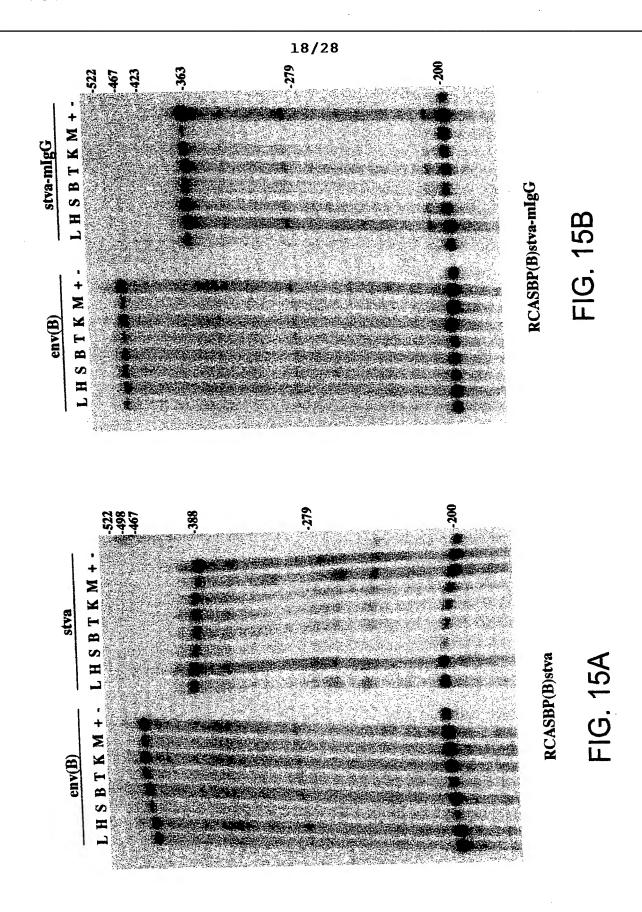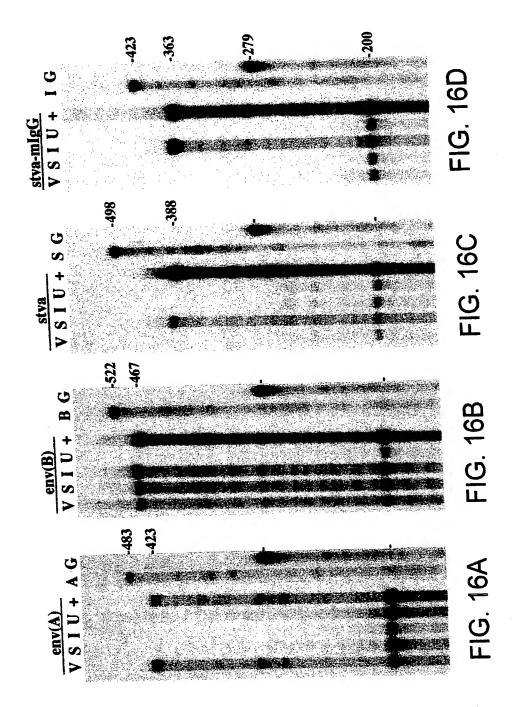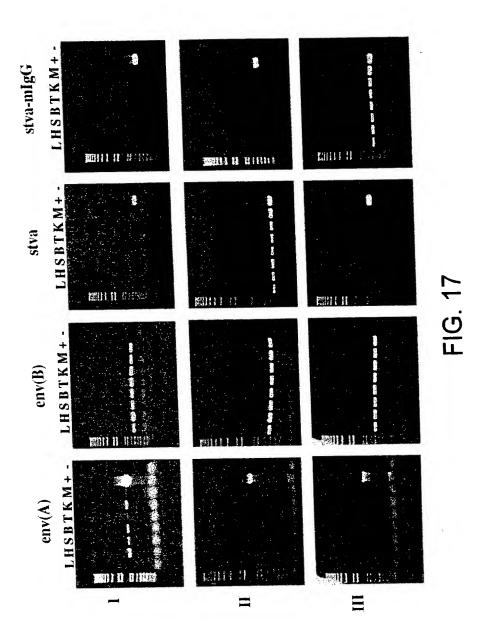
Figure 18

PERV 1.15

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AACCCTTATCTCTCACCTGGTTACTTACTGACTCCGGTACAGGTATTAATATTAACAGCACTCAAGGGGGAGGCTCCCTTG
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ACTCCGTGCTTACGGGTTTTACGTTTGCCCAGGGCCCCCAAATAATGAAGAATATTGTGGAAATCCTCAGGATTTCTTTT
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TCAAGGGTGGTTTGAGGGATGGTTCAACAGGTCTCCTTGGTTGGCTACCCTACTTTCTGCTTTAACAGGACCCTTAATAG
TCCTCCTCCTGTTACTCACAGTTGGGCCATGTATTATTAACAAGTTAATTGCCTTCATTAGAGAACGAATAAGTGCAGTC
CAGATCATGGTACTTAGACAACAGTACCAAAGCCCGTCTAGCAGAGAAGCTGGCCGCTAG

LamAl

TACTTCTTGGGGAAGACCCTGGGCTGCTAACTGGGTCTTGGCTGGTCCTAGTGAAAGGATGAAAATGCAACCTGACTCTC
CCAGAACCCAGGAAGTTAATAAGAAGCTCTAAATAATGAAAGGATGAAAATGCAACCTGACTCTCCCAGAACCCAGGAAG
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<120> Methods to inhibit infectious agent transmission associated
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     Ser Pro Asn Ser His Lys Pro Leu Ser Leu Thr Trp Leu Leu Thr Asp
              50                    55                    60
  25 Ser Gly Thr Gly Ile Asn Ile Asn Ser Thr Gln Gly Glu Ala Pro Leu
     65                    70                    75                    80
        Gly Thr Trp Trp Pro Glu Leu Tyr Val Cys Leu Arg Ser Val Ile Pro
                          85                    90                    95
        Gly Leu Asn Asp Gln Ala Thr Pro Pro Asp Val Leu Arg Ala Tyr Gly
30                    100                    105                    110
     Phe Tyr Val Cys Pro Gly Pro Pro Asn Asn Glu Glu Tyr Cys Gly Asn
                    115                    120                    125
     Pro Gln Asp Phe Phe Cys Lys Gln Trp Ser Cys Ile Thr Ser Asn Asp
                    130                    135                    140
  35 Gly Asn Trp Lys Trp Pro Val Ser Gln Gln Asp Arg Val Ser Tyr Ser
     145                    150                    155                    160
        Phe Val Asn Asn Pro Thr Ser Tyr Asn Gln Phe Asn Tyr Gly His Gly
                          165                    170                    175
        Arg Trp Lys Asp Trp Gln Gln Arg Val Gln Lys Asp Val Arg Asn Lys
40                    180                    185                    190
```

5

```
    Gln Ile Ser Cys His Ser Leu Asp Leu Asp Tyr Leu Lys Ile Ser Phe
            195                 200                 205

    Thr Glu Lys Gly Lys Gln Glu Asn Ile Gln Lys Trp Val Asn Gly Ile
            210                 215                 220

  5 Ser Trp Gly Ile Val Tyr Tyr Gly Gly Ser Gly Arg Lys Lys Gly Ser
    225                 230                 235                 240

    Val Leu Thr Ile Arg Leu Arg Ile Glu Thr Gln Met Glu Pro Pro Val
                    245                 250                 255

    Ala Ile Gly Pro Asn Lys Gly Leu Ala Glu Gln Gly Pro Pro Ile Gln
 10             260                 265                 270

    Glu Gln Arg Pro Ser Pro Asn Pro Ser Asp Tyr Asn Thr Thr Ser Gly
                275                 280                 285

    Ser Val Pro Thr Glu Pro Asn Ile Thr Ile Lys Thr Gly Ala Lys Leu
            290                 295                 300

 15 Phe Ser Leu Ile Gln Gly Ala Phe Gln Ala Leu Asn Ser Thr Thr Pro
    305                 310                 315                 320

    Glu Ala Thr Ser Ser Cys Trp Leu Cys Leu Ala Ser Gly Pro Pro Tyr
                    325                 330                 335

    Tyr Glu Gly Met Ala Arg Gly Gly Lys Phe Asn Val Thr Lys Glu His
 20             340                 345                 350

    Arg Asp Gln Cys Thr Trp Gly Ser Gln Asn Lys Leu Thr Leu Thr Glu
                355                 360                 365

    Val Ser Gly Lys Gly Thr Cys Ile Gly Met Val Pro Pro Ser His Gln
            370                 375                 380

 25 His Leu Cys Asn His Thr Glu Ala Phe Asn Arg Thr Ser Glu Ser Gln
    385                 390                 395                 400

    Tyr Leu Val Pro Gly Tyr Asp Arg Trp Trp Ala Cys Asn Thr Gly Leu
                405                 410                 415

    Thr Pro Cys Val Ser Thr Leu Val Phe Asn Gln Thr Lys Asp Phe Cys
 30             420                 425                 430

    Val Met Val Gln Ile Val Pro Arg Val Tyr Tyr Tyr Pro Glu Lys Ala
                435                 440                 445

    Val Leu Asp Glu Tyr Asp Tyr Arg Tyr Asn Arg Pro Lys Arg Glu Pro
            450                 455                 460

 35 Ile Ser Leu Thr Leu Ala Val Met Leu Gly Leu Gly Val Ala Ala Gly
    465                 470                 475                 480

    Val Gly Thr Gly Thr Ala Ala Leu Ile Thr Gly Pro Gln Gln Leu Glu
                485                 490                 495

    Lys Gly Leu Ser Asn Leu His Arg Ile Val Thr Glu Asp Leu Gln Ala
 40             500                 505                 510
```

6

```
     Leu Glu Lys Ser Val Ser Asn Leu Glu Glu Ser Leu Thr Ser Leu Ser
             515                 520                 525

     Glu Val Val Leu Gln Asn Arg Arg Gly Leu Asp Leu Leu Phe Leu Lys
             530                 535                 540

    5Glu Gly Gly Leu Cys Val Ala Leu Lys Glu Glu Cys Cys Phe Tyr Val
     545                 550                 555                 560

     Asp His Ser Gly Ala Ile Arg Asp Ser Met Ser Lys Leu Arg Glu Arg
                         565                 570                 575

     Leu Glu Arg Arg Arg Arg Glu Arg Glu Ala Asp Gln Gly Trp Phe Glu
    10             580                 585                 590

     Gly Trp Phe Asn Arg Ser Pro Trp Met Thr Thr Leu Leu Ser Ala Leu
                 595                 600                 605

     Thr Gly Pro Leu Val Val Leu Leu Leu Leu Leu Thr Val Gly Pro Cys
             610                 615                 620

    15Leu Ile Asn Arg Phe Val Ala Phe Val Arg Glu Arg Val Ser Ala Val
     625                 630                 635                 640

     Gln Ile Met Val Leu Arg Gln Gln Tyr Gln Gly Leu Leu Ser Gln Gly
                 645                 650                 655

     Glu Thr Asp Leu
    20             660


     <210> 5
     <211> 638
     <212> PRT
    25<213> Porcine endogenous retrovirus


     <400> 5
     Met His Pro Thr Leu Asn Arg Arg His Leu Pro Ile Arg Gly Gly Lys
     1               5                   10                  15

    30Pro Lys Arg Leu Lys Ile Pro Leu Ser Phe Ala Ser Ile Ala Trp Phe
                 20                  25                  30

     Leu Thr Leu Ser Ile Thr Ser Gln Thr Asn Gly Met Arg Ile Gly Asp
                 35                  40                  45

     Ser Leu Asn Ser His Lys Pro Leu Ser Leu Thr Trp Leu Ile Thr Asp
    35   50                  55                  60

     Ser Gly Thr Gly Ile Asn Ile Asn Asn Thr Gln Gly Glu Ala Pro Leu
     65                  70                  75                  80

     Gly Thr Trp Trp Pro Asp Leu Tyr Val Cys Leu Arg Ser Val Ile Pro
                 85                  90                  95

    40Ser Leu Thr Ser Pro Pro Asp Ile Leu His Ala His Gly Phe Tyr Val
```

7

```
                    100                    105                      110
       Cys Pro Gly Pro Pro Asn Asn Gly Lys His Cys Gly Asn Pro Arg Asp
                    115                    120              125
       Phe Phe Cys Lys Gln Trp Asn Cys Val Thr Ser Asn Asp Gly Tyr Trp
  5    130                    135                    140
       Lys Trp Pro Thr Ser Gln Gln Asp Arg Val Ser Phe Ser Tyr Val Asn
       145                    150                    155                160
       Thr Tyr Thr Ser Ser Gly Gln Phe Asn Tyr Leu Thr Trp Ile Arg Thr
                           165                    170              175
  10Gly Ser Pro Lys Cys Ser Pro Ser Asp Leu Asp Tyr Leu Lys Ile Ser
                    180                    185              190
       Phe Thr Glu Lys Gly Lys Gln Glu Asn Ile Leu Lys Trp Val Asn Gly
                    195                    200              205
       Met Ser Trp Gly Met Val Tyr Tyr Gly Gly Ser Gly Lys Gln Pro Gly
  15    210                    215                    220
       Ser Ile Leu Thr Ile Arg Leu Lys Ile Asn Gln Leu Glu Pro Pro Met
       225                    230                    235                240
       Ala Ile Gly Pro Asn Thr Val Leu Thr Gly Gln Arg Pro Pro Thr Gln
                           245                    250              255
  20Gly Pro Gly Pro Ser Ser Asn Ile Thr Ser Gly Ser Asp Pro Thr Glu
                    260                    265              270
       Ser Ser Ser Thr Thr Lys Met Gly Ala Lys Leu Phe Ser Leu Ile Gln
                    275                    280              285
       Gly Ala Phe Gln Ala Leu Asn Ser Thr Thr Pro Glu Ala Thr Ser Ser
  25    290                    295                    300
       Cys Trp Leu Cys Leu Ala Ser Gly Pro Pro Tyr Tyr Glu Gly Met Ala
       305                    310                    315                320
       Arg Arg Gly Lys Phe Asn Val Thr Lys Glu His Arg Asp Gln Cys Thr
                           325                    330              335
  30Trp Gly Ser Gln Asn Lys Leu Thr Leu Thr Glu Val Ser Gly Lys Gly
                    340                    345              350
       Thr Cys Ile Gly Lys Val Pro Pro Ser His Gln His Leu Cys Asn His
                    355                    360              365
       Thr Glu Ala Phe Asn Gln Thr Ser Glu Ser Gln Tyr Leu Val Pro Gly
  35    370                    375                    380
       Tyr Asp Arg Trp Trp Ala Cys Asn Thr Gly Leu Thr Pro Cys Val Ser
       385                    390                    395                400
       Thr Leu Val Phe Asn Gln Thr Lys Asp Phe Cys Ile Met Val Gln Ile
                           405                    410              415
  40Val Pro Arg Val Tyr Tyr Tyr Pro Glu Lys Ala Ile Leu Asp Glu Tyr
```

8

```
                 420                   425                   430
    Asp Tyr Arg Asn His Arg Gln Lys Arg Glu Pro Ile Ser Leu Thr Leu
             435                   440                   445
    Ala Val Met Leu Gly Leu Gly Val Ala Ala Gly Val Gly Thr Gly Thr
 5       450                   455                   460
    Ala Ala Leu Val Thr Gly Pro Gln Gln Leu Glu Thr Gly Leu Ser Asn
    465                   470                   475                   480
    Leu His Arg Ile Val Thr Glu Asp Leu Gln Ala Leu Glu Lys Ser Val
                     485                   490                   495
10Ser Asn Leu Glu Glu Ser Leu Thr Ser Leu Ser Glu Val Val Leu Gln
                 500                   505                   510
    Asn Arg Arg Gly Leu Asp Leu Leu Phe Leu Lys Glu Gly Gly Leu Cys
             515                   520                   525
    Val Ala Leu Lys Glu Glu Cys Cys Phe Tyr Val Asp His Ser Gly Ala
15       530                   535                   540
    Ile Arg Asp Ser Met Asn Lys Leu Arg Glu Arg Leu Glu Lys Arg Arg
    545                   550                   555                   560
    Arg Glu Lys Glu Thr Thr Gln Gly Trp Phe Glu Gly Trp Phe Asn Arg
                     565                   570                   575
20Ser Leu Trp Leu Ala Thr Leu Leu Ser Ala Leu Thr Gly Pro Leu Ile
                 580                   585                   590
    Val Leu Leu Leu Leu Leu Thr Val Gly Pro Cys Ile Ile Asn Lys Leu
             595                   600                   605
    Ile Ala Phe Ile Arg Glu Arg Ile Ser Ala Val Gln Ile Met Val Leu
25       610                   615                   620
    Arg Gln Gln Tyr Gln Ser Pro Ser Ser Arg Glu Ala Gly Arg
    625                   630                   635
```

```
    <210> 6
30<211> 704
    <212> DNA
    <213> Porcine endogenous retrovirus

    <400> 6
35aatgaaagga tgaaaatgca acctgactct cccagaaccc aggaagttaa taagaagctc      60
    taaatgccct cgaattccag accctgttcc ctataggtaa aagatcatac ttttttgctgt    120
    tttaaaatat gctttctgct ctgtacaaaa ctttgtggaa ggggaaaaac aggcccctga     180
    gtatgtgcct ctatgcttga aacttcttga aactgctcct aactgcttgt ttggcttctg     240
    taaacctgct tgcataagat aaaaagagga gaagtcaatt gcctaacgga ccccagtaag     300
40atcgggtgta ccacaaaatg ttgaaacaca tatcttggtg acaacatgtc tcccccaccc     360
```

9

```
cgaaacatgc gcaaatgtgt aactctaaaa caatttaaat taattggtcc acgaagcgcg    420
ggctctcgaa gttttaaatt gactggtttg tgatattttg aaatgattgg tttgtaaagc    480
gcgggctttg ttgtgaaccc cataaaagct gtcccgactc cacactcggg gccgcagtcc    540
tctacccctg cgtggtgtac gactgtgggc cccagcgcgc ttggaataaa aatcctcttg    600
5ctgtttgcat caagaccgct tctcgtgagt gattaagggg agtcgccttt tccgagcctg    660
gaggttcttt ttgctagtct tacatttggg ggctcgtccg ggat                     704
```

```
<210> 7
<211> 633
10<212> DNA
<213> Porcine endogenous retrovirus
```

```
<400> 7
aatgaaagga tgaaaataca acctaagcta atgagaagct taaaattgtt ctgaattcca     60
15gagtttgttc cttataggta aaagattagg tttttttgctg ttttaaaata tgcggaagta    120
aaataggccc tgagtacatg tctctaggca tgaaacttct tgaaactatt tgagataaca    180
agaaaaggga gtttctaact gcttgtttag cttctgtaaa actggttgcg ccataaagat    240
gttgaaatgt tgatacacat atcttggtga caacatgtct cccccacccc gaaacatgcg    300
caaatgtgta actctaaaac aatttaaatt aattggtcca cgaagcgcgg gctctcgaag    360
20ttttaaattg actggtttgt gatattttga aatgattggt ttgtaaagcg cgggctttgt    420
tgtgaacccc ataaaagctg tcccgactcc acactcgggg ccgcagtcct ctacccctgc    480
gtggtgtacg actgtgggcc ccagcgcgct tggaataaaa atcctcttgc tgtttgcatc    540
aagaccgctt ctcgtgagtg attaagggga gtcgcctttt ccgagcctgg aggttctttt    600
tgctggtctt acatttgggg gctcgtccgg gat                                 633
```

25
```
<210> 8
<211> 20
<212> DNA
<213> Porcine endogenous retrovirus
```
30
```
<400> 8
tggaaagatt ggcaacagcg                                                 20
```

```
<210> 9
35<211> 20
<212> DNA
<213> Porcine endogenous retrovirus
```

```
<400> 9
40agtgatgtta ggctcagtgg                                               20
```

10

```
<210> 10
<211> 20
<212> DNA
<213> Porcine endogenous retrovirus
```
5
```
<400> 10
ttctcctttg tcaattccgg                                                            20
```

```
<210> 11
<211> 20
<212> DNA
<213> Porcine endogenous retrovirus
```
10
```
<400> 11
tactttatcg ggtcccactg                                                            20
```
15

```
<210> 12
<211> 20
<212> DNA
<213> Porcine endogenous retrovirus
```
20
```
<400> 12
ctgacctgga ttagaactgg                                                            20
```
25
```
<210> 13
<211> 20
<212> DNA
<213> Porcine endogenous retrovirus
```
30
```
<400> 13
atgttagagg atggtcctgg                                                            20
```

```
<210> 14
<211> 22
<212> DNA
<213> Porcine endogenous retrovirus
```
35
```
<400> 14
acctcgagac tcggtggaag gg                                                         22
```
40

11

```
<210> 15
<211> 24
<212> DNA
<213> Porcine endogenous retrovirus

<400> 15
ctgggttctg ggagggttag gttg                                      24


<210> 16
<211> 24
<212> DNA
<213> Porcine endogenous retrovirus


<400> 16
acgtactgga ggagggtcac ctga                                      24


<210> 17
<211> 24
<212> DNA
<213> Porcine endogenous retrovirus


<400> 17
gtcccgaacc cttataacct cttg                                      24


<210> 18
<211> 1980
<212> DNA
<213> Porcine endogenous retrovirus


<400> 18
atgcatccca cgttaagccg gcgccacctc ccgattcggg gtggaaagcc gaaaagactg    60
aaaatcccct taagcttcgc ctccatcgcg tggttcctta ctctgtcaat aactcctcaa   120
gttaatggta aacgccttgt ggacagcccg aactcccata aaccccttatc tctcacctgg   180
ttacttactg actccggtac aggtattaat attaacagca ctcaaggggga ggctcccttg   240
gggacctggt ggcctgaatt atatgtctgc cttcgatcag taatccctgg tctcaatgac   300
caggccacac cccccgatgt actccgtgct tacgggtttt acgtttgccc agggcccca   360
aataatgaag aatattgtgg aaatcctcag gatttcttttt gcaagcaatg gagctgcgta   420
acttctaatg atgggaattg gaaatggcca gtctctcagc aagacagagt aagttactct   480
tttgttaaca atcctaccag ttataatcaa tttaattatg gccatgggag atggaaagat   540
tggcaacagc gggtacaaaa agatgtacga aataagcaaa taagctgtca ttcgttagac   600
```

12

```
   ctagattact taaaaataag tttcactgaa aaaggaaaac aagaaaatat tcaaaagtgg      660
   gtaaatggta tgtcttgggg aatagtgtac tatagaggct ctgggagaaa gaaaggatct      720
   gttctgacta ttcgcctcag aatagaaact cagatggaac ctccggttgc tataggacca      780
   aataagggtt tggccgaaca aggacctcca atccaagaac agaggccatc tcctaacccc      840
 5tctgattaca atacaacctc tggatcagtc cccactgagc ctaacatcac tattaaaaca      900
   ggggcgaaac tttttaacct catccaggga gcttttcaag ctcttaactc cacgactcca      960
   gaggctacct cttcttgttg ctttgcttta gcttcgggcc caccttacta tgagggaatg     1020
   gctagaggag ggaaattcaa tgtgacaaag gaacatagag accaatgtac atggggatcc     1080
   caaaataagc ttacccttac tgaggtttct ggaaaaggca cctgcatagg gatggttccc     1140
10ccatcccacc aacacctttg taaccacact gaagccttta atcgaacctc tgagagtcag     1200
   tatctggtac ctggttatga caggtggtgg gcatgtaata ctggattaac cccttgtgtt     1260
   tccaccttgg ttttcaacca aactaaagac ttttgcgtta tggtccaaat tgtcccccgg     1320
   gtgtactact atcccgaaaa agcagtcctt gatgaatatg actatagata taatcggcca     1380
   aaaagagagc ccatatccct gacactagct gtaatgctcg gattgggagt ggctgcaggc     1440
15gtgggaacag gaacggctgc cctaatcaca ggaccgcaac agctggagaa aggacttagt     1500
   aacctacatc gaattgtaac ggaaaatctc caagccctag aaaaatctgt cagtaacctg     1560
   gaggaatccc taacctcctt atctgaagtg gttctacaga acagaagggg gttagatctg     1620
   ttatttctaa aagaaggagg attatgtgta gccttaaagg aggaatgctg tttttatgtg     1680
   gatcattcag gggccatcag agactccatg aacaagctta gagaaaggtt ggagaagcgt     1740
20cgaagggaaa aggaaactac tcaagggtgg tttgagggat ggttcaacag gtctccttgg     1800
   ttggctaccc tactttctgc tttaacagga cccttaatag tcctcctcct gttactcaca     1860
   gttgggccat gtattattaa caagttaatt gccttcatta gagaacgaat aagtgcagtc     1920
   cagatcatgg tacttagaca acagtaccaa agcccgtcta gcagagaagc tggccgctag     1980
```

25<210> 19

  <211> 7362

  <212> DNA

  <213> Porcine endogenous retrovirus

30<400> 19

```
   tacttcttgg ggaagaccct gggctgctaa ctgggtcttg gctggtccta gtgaaaggat       60
   gaaaatgcaa cctgactctc ccagaaccca ggaagttaat aagaagctct aaataatgaa      120
   aggatgaaaa tgcaacctga ctctcccaga acccaggaag ttaataagaa gctctaaatg      180
   ccctcgaatt ccagaccctg ttccctatag gtaaaagatc atactttttg ctgttttagg      240
35gcttgctttc tgctctgtac aaaactttgt ggaaggggaa aaacaggccc ctgagtatgt      300
   gcctctatgc ttgaaacttc ttgaaactgc tcctaactgc ttgtttggct tctgtaaacc      360
   tgcttgcata agataaaaag aggagaagtc aattgcctaa cggaccccag taagatcggg      420
   tgtaccacaa aatgttgaaa cacatatctt ggtgacaaca tgtctccccc accccgaaac      480
   atgcgcaaat gtgtaactct aaaacaattt aaattaattg tccacgaag cgcgggctct      540
40cgaagtttta aattgactgg tttgtgatat tttgaaatga ttggtttgta aagcgcgggc      600
```

13

```
tttgttgtga accccataaa agctgtcccg actccacact cggggccgca gtcctctacc    660
cctgcgtggt gtacgactgt gggccccagc gcgcttggaa taaaaatcct cttgctgttt    720
gcatcaagac cgcttctcgt gagtgattaa ggggagtcgc cttttccgag cctggaggtt    780
ctttttgcta gtcttacatt tgggggctcg tccgggatct gtcgcggcca cccctaacac    840
5ccgagaaccg acttggaggt aaaaaggatc ctctttttaa cgtgtatgca tgtaccggcc    900
ggcgtctctg ttctgagtgt ctgttttcag tggtgcgcgc tttcggtttg cagctgtcct    960
ctcagaccgt aaggactggg ggactgtgat cagcagacgt gctaggagga tcacaggctg   1020
ccaccctggg ggacgccccg ggaggtgggg agagccaggg acgcctggtg gtctccttct   1080
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ctgttttgtg tgtctttgtc ttgtgcgtcc ttgtctacag ttttaatatg ggacagacgg   1260
tgacgacccc tcttagtttg actctcgacc attggactga agttaaatcc agggctcata   1320
atttgtcagt tcaggttaag aagggacctt ggcagacttt ctgtgtctct gaatggccga   1380
cattcgatgt tggatggcca tcagagggga cctttaattc tgagattatc ctggctgtta   1440
15aagcaattat ttttcagact ggacccggct ctcatcccaa tcaggagccc tatatcctta   1500
cgtggcaaga tttggcagag gatcctccgc catgggttaa accttggctg aataagccaa   1560
gaaagccagg tccccgaatt ctggctcttg gagagaaaaa caaacactcg gctgaaaaag   1620
tcaagccctc tcctcatatc taccccgaga ttgaggagcc gccggcttgg ccggaacccc   1680
aatctgttcc cccacccccct tatctggcac agggtgctgc gaggggaccc tctgcccctc    1740
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cggagcggac agacgagatc gcgacattac cgctgcgcac gtacggccct cccacaccgg   1860
ggggccaatt gcagcccctc cagtattggc ccttttcttc tgcagatctc tataattgga   1920
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ttatgttctc tcaccagcct acttgggatg attgtcaaca gctgctgcag acactcttca   2040
25caaccgagga gcgagagaga attctgttag aggctagaaa aaatgttcct ggggccgacg   2100
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30acaccccttt tgatcccacc tcagaggccc aaaaagcctc agtggctttg gcctttatag   2400
gacagtcagc cttggatatt agaaagaagc ttcagagact ggaagggtta caggaggctg   2460
agttacgtga tctagtgaag gaggcagaga aagtatatta caaaagggag acagaagaag   2520
aaagggaaca aagaaaagag agagaaagag aggaaaggga ggaaagacgt aataaacggc   2580
aagagaagaa tttgactaag atcttggctg cagtggttga agggaaaagc aatacggaaa   2640
35gagagagaga ttttaggaaa attaggtcag ccctagaca gtcagggaac ctgggcaata    2700
ggaccccact cgacaaggac caatgtgcat attgtaaaga aaaggacac tgggcaagga    2760
actgccccaa gaagggaaac aaaggactga aggtcttagc tctgaaagaa gataaagact   2820
agggaagacg gggttcggac cccctccccg agcccagggt aactttgaag gtggaggggc   2880
aaccagttga gttcctggtt gataccggag cgaaacattc agtgctacta cagccattag   2940
40gaaaactaaa agataaaaaa tcctgggtga tgggtgccac agggcaacaa cagtatccat   3000
```

14

```
    ggactacccg aagaacagtt gacttgggag tgggacgggt aacccactcg tttctggtca    3060
    tacctgagtg cccagcaccc ctcttaggta gagacttatt gaccaagatg ggagcacaaa    3120
    tttcttttga acaagggaaa ccagaagtgt ctgcaaataa caaacctatc actgtgttga    3180
    ccctccaatt agatgacgaa tatcgactat actctcccct agtaaagcct gatcaaaata    3240
  5 tacaattctg gttggaacag tttccccaag cctgggcaga aaccgcaggg atgggtttgg    3300
    caaagcaagt tcccccacaa gttattcaac tgaaggccag tgccacacca gtgtcagtca    3360
    gacagtaccc cttgagtaaa gaagctcaag aaggaattcg ccgcatgtc caaagattaa     3420
    tccaacaggg catcctagtt cctgtccaat ctccctggaa tactcccctg ctaccggtta    3480
    gaaagcctgg gactaatgac tatcgaccag tacaggactt gagagaggtc aataaacggg    3540
 10 tgcaggatat acacccaaca gtcccgaacc cttataacct cttgtgtgct ctcccacccc    3600
    aacggagctg gtatacagta ttggacttaa aggatgcctt tttctgcctg agattacacc    3660
    ccactagcca accactttt gccttcgaat ggagagatcc aggtacggga agaaccgggc     3720
    agctcacctg gacccgactg ccccaagggt tcaagaactc cccgaccatc tttgacgaag    3780
    ccctacacag agacctggcc aacttcagga tccaacaccc tcaggtgacc ctcctccagt    3840
 15 acgtggatga cctgcttctg gcgggagcca ccaaacagga ctgcttagaa ggcacgaagg    3900
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    gcaggagaga ggtaacatac ttggggtaca gtttgcggga cgggcagcga tggctgacgg    4020
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21

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22

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24

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25

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26

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INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7   C12N15/48   C12N15/62   C07K14/15   C07K19/00   A61K48/00
         C12Q1/68   C07K16/10   C12N7/01

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7   C12N   C07K   A61K   C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	AKIYOSHI D. E. ET AL.: "Identification of a full-length cDNA for an endogenous retrovirus of miniature swine" JOURNAL OF VIROLOGY, vol. 72, no. 5, May 1998 (1998-05), pages 4503-4507, XP002126425 ISSN: 0022-538X	15-20
Y	the whole document	1-14, 21-28, 30,32-39

	-/--	

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 September 2000	04/10/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340–2040, Tx. 31 651 epo nl, Fax: (+31-70) 340–3016	Mandl, B

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Form PCT/ISA/210 (second sheet) (July 1992)

Int   ational Application No

PCT/US 00/14296

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	VANBROCKLIN M. ET AL.: "Expression of a murine leukemia virus Gag-Escherichia coli RNase HI fusion polyprotein significantly inhibits virus spread." JOURNAL OF VIROLOGY, vol. 71, no. 4, 1997, pages 3312-3318, XP002148090 ISSN: 0022-538X the whole document	1-14, 21-28, 32-39
X	WO 98 53104 A (MEDICAL RES COUNCIL ;STOYE JONATHAN PAUL (GB); WEISS ROBIN ANTHONY) 26 November 1998 (1998-11-26)	29,31
Y	the whole document	30
A	NATSOULIS G. ET AL.: "NEW ANTIVIRAL STRATEGY USING CAPSID-NUCLEASE FUSION PROTEINS" NATURE, vol. 352, no. 6336, 15 August 1991 (1991-08-15), pages 632-635, XP000608400 ISSN: 0028-0836 the whole document	15-28
A	SUN L.-Q. ET AL.: "Ribozyme-mediated suppression of Moloney murine leukemia virus and human immunodeficiency virus type I replication in permissive cells." PROC. NATL. ACAD. SCI. USA, vol. 91, October 1994 (1994-10), pages 9715-9719, XP002148091 the whole document	13
A	WO 96 32494 A (UNIV ALABAMA RES FOUND) 17 October 1996 (1996-10-17) the whole document	15-28
A	PATIENCE C. ET AL.: "INFECTION OF HUMAN CELLS BY AN ENDOGENOUS RETROVIRUS OF PIGS" NATURE MEDICINE, vol. 3, no. 3, 1 March 1997 (1997-03-01), pages 282-286, XP002037074 ISSN: 1078-8956 the whole document	1-39

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FURTHER INFORMATION CONTINUED FROM   PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-28,32-39

> A method to inhibit or prevent infectious agent transmission in a mammalian transplant recipient; an isolated and purified nucleic acid molecule comprising at least a portion of a pig endogenous retrovirus, the corresponding polypeptides and methods of using them; a method to detect human tropic pig endogenous retroviruses; a fusion protein comprising a capsid or envelope protein of PERV and a degradative enzyme, the corresponding nucleic acid and a host cell comprising it.

2. Claims: 29-31

> An antibody that specifically bings pig endogenous retrovirus.

INTERNATIONAL SEARCH REPORT

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9853104	A	26-11-1998	AU	7442698 A	11-12-1998
			EP	0981651 A	01-03-2000
			GB	2333102 A	14-07-1999
			US	6100034 A	08-08-2000
WO 9632494	A	17-10-1996	AU	5482896 A	30-10-1996
			CA	2230925 A	17-10-1996
			EP	0873415 A	28-10-1998
			US	6001985 A	14-12-1999